On the rules of co-existence between sheep, cattle, and OvHV-2

Abstract

Malignant catarrhal fever (MCF) is a lethal disease of ruminants and swine, characterized by vasculitis, necrosis, and accumulation of activated, dysregulated cytotoxic lymphocytes in various tissues. It is transmitted from so-called “reservoir” animal species to “indicator” animal species. Ovine gammaherpesvirus 2 (OvHV-2) is one of several causative agents of MCF, circulating among all sheep breeds worldwide, which represent the “reservoir” of this virus. Natural infection of sheep proceeds without clinical symptoms. The disease is characterized by proliferation of cytotoxic T-cells, which form perivascular infiltrates of lymphoid as well as non-lymphoid organs, leading to necrosis and tissue destruction. However, little is known about the immunopathogenic pathways leading to disease. Moreover, at the time when this work was initiated, only limited data were available for the situation in sheep. Therefore, we first analyzed the cell populations targeted by the virus during natural infection of lambs, since the range of targeted cells may be important for the pathogenesis, especially if it is different in sheep and indicator animals, respectively. In most sheep we found a first peak of viral DNA in CD4+ T-helpercells, followed by a second peak in CD8+ cytotoxic T-cells. Both, CD4+ as well as CD8+ cells are also found to harbor viral DNA in lymphocytes of cattle with MCF. From this finding we suggest that rather a speciesspecific fine tuning of viral gene expression than the types of targeted cells may be accountable for the disease. The second question addressed in this work was whether production of viral particles leading to cell lysis is among the pathological factors of MCF. Diseases due to gammaherpesvirus infections are mostly associated to latent infection, during which only a limited number of viral proteins are expressed. These are important to tie the viral DNA to host chromosomes, ensuring synchronous replication of viral DNA with cellular DNA, to provide viral DNA to each daughter cell and likely render the host cell increased resistance against apoptosis. However, persistent infection with Human herpesvirus 8 (HHV-8) as an exception is depending on a mixture of latently and lytically infected cells. To address whether this may be the case during MCF as well, we produced antisera against structural viral proteins and tested several tissue samples of experimentally infected rabbits therewith. We detected viral proteins in epithelial and M-cells of the appendix of infected animals. Interestingly, in situ hybridization revealed viral RNA in the infected epithelial cells but not in M-cells. These data suggest that active OvHV-2 replication may play a role in the pathogenesis of the disease. Thirdly, we tested the gene expression patterns of OvHV-2 and the relative abundances of host cell transcripts in lymphocytes of diseased cattle to identify pathways possibly involved in the pathogenesis of MCF. To this, host and virus gene expression patterns were analyzed by microarrray. Only two regions of the viral genome were found to be transcriptionally active, one encoding a latency-associated nuclear antigen, which can be found during latency of other gammaherpesviruses as well. The other with no predicted open reading frame, which may represent micro RNA (miRNA). So far miRNA was not discussed as a pathological factor of MCF and opens completely new attempts. As could be expected, a large number of host genes related to inflammation, lymphocyte activation, cell proliferation and apoptosis were found to be at different abundances compared to healthy animals. One of these transcripts with decreased expression was IL-2. Since the phenotype of mice with IL-2 deficiency perfectly matches the clinical signs of MCF, we assume that IL-2 deficiency may play an important role in the development of disease.

On the Rules of Co-existence between Sheep, Cattle, and OvHV-2

PhD Thesis

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1 Abstract

Malignant catarrhal fever (MCF) is a lethal disease of ruminants and swine, characterized by vasculitis, necrosis, and accumulation of activated, dysregulated cytotoxic lymphocytes in various tissues. It is transmitted from so-called “reservoir” animal species to “indicator” animal species. Ovine gammaherpesvirus 2 (OvHV-2) is one of several causative agents of MCF, circulating among all sheep breeds worldwide, which represent the “reservoir” of this virus. Natural infection of sheep proceeds without clinical symptoms. The disease is characterized by proliferation of cytotoxic T-cells, which form perivascular infiltrates of lymphoid as well as non-lymphoid organs, leading to necrosis and tissue destruction. However, little is known about the immunopathogenic pathways leading to disease. Moreover, at the time when this work was initiated, only limited data were available for the situation in sheep. Therefore, we first analyzed the cell populations targeted by the virus during natural infection of lambs, since the range of targeted cells may be important for the pathogenesis, especially if it is different in sheep and indicator animals, respectively. In most sheep we found a first peak of viral DNA in CD4+ T-helpercells, followed by a second peak in CD8+ cytotoxic T-cells. Both, CD4+ as well as CD8+ cells are also found to harbor viral DNA in lymphocytes of cattle with MCF. From this finding we suggest that rather a species-specific fine tuning of viral gene expression than the types of targeted cells may be accountable for the disease. The second question addressed in this work was whether production of viral particles leading to cell lysis is among the pathological factors of MCF. Diseases due to gammaherpesvirus infections are mostly associated to latent infection, during which only a limited number of viral proteins are expressed. These are important to tie the viral DNA to host chromosomes, ensuring synchronous replication of viral DNA with cellular DNA, to provide viral DNA to each daughter cell and likely render the host cell increased resistance against apoptosis. However, persistent infection with Human herpesvirus 8 (HHV-8) as an exception is depending on a mixture of latently and lytically infected cells. To address whether this may be the case during MCF as well, we produced antisera against structural viral proteins and tested several tissue samples of experimentally infected rabbits therewith. We detected viral proteins in epithelial and M-cells of the appendix of infected animals. Interestingly, in situ hybridization revealed viral RNA in the infected epithelial cells but not in M-cells. These data suggest that active OvHV-2 replication may play a role in the pathogenesis of the disease. Thirdly, we tested the gene expression patterns of OvHV-2 and the relative abundances of host cell transcripts in lymphocytes of diseased cattle to identify pathways possibly involved in the pathogenesis of MCF. To this, host and virus gene expression patterns were analyzed by microarrray. Only two regions of the viral genome were found to be transcriptionally active, one encoding a latency-associated nuclear antigen, which can be found during latency of other gammaherpesviruses as well. The other with no predicted open reading frame, which may represent micro RNA (miRNA). So far miRNA was not discussed as a pathological factor of MCF and opens completely new attempts. As could be expected, a large number of host genes related to inflammation, lymphocyte activation, cell proliferation and apoptosis were found to be at different abundances compared to healthy animals. One of these transcripts with decreased expression was IL-2. Since the phenotype of mice with IL-2 deficiency perfectly matches the clinical signs of MCF, we assume that IL-2 deficiency may play an important role in the development of disease.

2 Introduction

Many problems in veterinary medicine due to viral infections have been solved or at least reduced thanks to strategies involving vaccination and/or eradication. None of this is true in the case of malignant catarrhal fever (MCF), which is associated to infection of particular animal species with specific gammaherpesviruses.

Herpesviruses are large, enveloped viruses with an icosahedral capsid containing a double-stranded DNA genome of 125-290 kbp. Taxonomically, the herpesviruses form the order *herpesvirales*, which can be subdivided into three families, i.e. the *herpesviridae*, the *alloherpesviridae* (herpesviruses of fish and frogs), and the *malacoherpesviridae* (herpesviruses of bivalves)[1]. The *herpesviridae* family comprises the herpesviruses of mammals, birds, and reptiles and is at present of highest medical and veterinary medical interest. Distinct biological properties have allowed to further divide this family into three subfamilies, i.e. the *alphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae*. Numerous members of the alphaherpesviruses and the gammaherpesviruses are of very high importance in veterinary medicine.

Establishment of latency and reactivation is probably the most important biological feature of the herpesvirus life cycle. Latency (Latin: *latens*, to be hidden) is defined as a persistent state of the infection, during which productive viral replication does not take place. A latently infected cell harbors the entire viral genome mostly in an episomal, circular, and almost inactive state. Such a cell is, therefore, almost invisible for the immune system and the infection can persist for as long as latently infected cells survive within the originally infected organism. Periodically, the herpesviruses reactivate from latency to undergo replicative cycles with the aim to be transmitted to new hosts, thus, perpetuating their existence in nature. Based on these features, herpesviruses establish their main reservoir in latently infected organisms.

A hallmark in the distinction between alphaherpesviruses and gammaherpesviruses is associated to the balance between latency and replicative cycle achieved upon infection of a susceptible cell. In general, alphaherpesviruses go primarily through the replicative cycle upon infection, which leads to efficient destruction of the cell and, consequently, the underlying tissue. Diseases associated to alphaherpesvirus infections can, therefore, be generally explained by tissue destruction due to lytic virus replication. In contrast, the gammaherpesviruses have a tendency to establish immediately a latent state of infection, which leads to a less pronounced tissue damage. The replicative cycle seems to be entered only rarely, only over short periods, and only under specific circumstances. While the alphaherpesviruses preferentially establish latency in long-living cells, for example neurons, the gammaherpesviruses have a preference for latency in the shorter-living lymphocytes. Therefore, the requirements to maintain latency are different between the members of the two subfamilies. In neurons, which do not divide and which are maintained for almost the entire lifespan, very little viral contribution may be required for maintaining latency. Consequently, only little viral activity can be observed throughout alphaherpesvirus latency. In contrast, it is more demanding to maintain a latent presence in dividing and maturing cells, such as lymphocytes, which have but a limited lifespan. Therefore, the latent state of the gammaherpesviruses relies strongly on certain viral activities, which, however, must not be recognizable for the immune system. It is easy to perceive that this latter strategy is prone to crisis because it demands a perfect balance between the actions of the virus and its host. Since lymphocytes distribute all over the organism, the latent gammaherpesvirus is equally dispersed. Once either the virus or the host breaks this equilibrium, a widely dispersed immune reaction may initiate, which may cause a severe problem for the entire organism.

This explains why diseases due to gammaherpesviruses may be linked with latent forms of the infection.

MCF is a frequently lethal, immunopathological, viral disease, which can be caused by several distinct gammaherpesviruses, and which is transmitted from so-called "reservoir" animal species to so-called "indicator" animal species. The animal species, in which the viruses have formed their reservoir(s), are fully susceptible to the viral infection but do not succumb to MCF. Therefore, the virus can freely circulate among them without causing harm. In contrast, members of the indicator animal species are usually not infected by the MCF-associated viruses, although they are susceptible to infection. While infection in those animals leads only rarely to successful replication and further transmission of the virus, some poorly understood pathogenetic mechanisms lead to the development of MCF in the accidentally infected members of the indicator species.

The present work has been performed to shed more light into the pathogenesis of MCF and one of its causative viruses, the Ovine gammaherpesvirus 2 (OvHV-2).

2.1 Gammaherpesviruses

Taxonomically, the gammaherpesviruses are subdivided into four genera, i.e. *lymphocryptovirus*, *rhadinovirus*, *macavirus*, and *percavirus* [1]. As a rule of thumb, these viruses are well adapted to their respective hosts. Most often, infection proceeds without overt clinical disease. For example, Epstein-Barr virus (EBV), the prototype of the lymphocryptoviruses, has established its reservoir in humans. Disease due to EBV occurs mainly in immunosuppressed people, although genetic and racial predisposition may play a role. Similarly, Human herpesvirus 8 (HHV-8), the prototype of the rhadinoviruses, can establish lifelong asymptomatic infection in humans. However, it is not fully elucidated whether there is a natural HHV-8-reservoir in a species other than humans. In immuno-suppressed hosts HHV-8 causes diseases such as Kaposi Sarcoma (KS), body cavity-based lymphoma, primary effusion lymphoma (PEL), and multicentric Castleman-syndrome (reviewed by [2]). Models for human rhadinovirus-associated diseases have been established with murine gammaherpesvirus 68 (MHV-68) and simian herpesvirus 2 (SaHV-2). However, other than its name suggests MHV-68 is not a mouse gammaherpesvirus but rather a virus of bank voles. Similarly, SaHV-2 has its reservoir in squirrel monkeys, not in primates, which are used as a disease model. However, interesting results may have emerged from these models, the question remains as to how accurate they reflect the human diseases. The designation "*Maca*" is abbreviated from malignant catarrhal fever and the genus *macavirus* comprises, consequently, a number of viruses from cloven-hoofed animals, which have either been detected in association with MCF, or which have high sequence similarity to those viruses. Known agents of MCF are alcelaphine herpesvirus 1 (AlHV-1; wildebeest-associated), AlHV-2 (hartebeest-associated), caprine herpesvirus 2 (CpHV-2; goat-associated), and ovine herpesvirus 2 (OvHV-2; sheep-associated). Characteristically, these viruses do not cause a natural disease in their reservoir hosts but may naturally cause MCF upon transmission to animals of the indicator host type. Thus, studying those viruses addresses a veterinary medical problem and addressing questions concerning the pathogenesis of MCF may be asked in a natural context.

All gammaherpesviruses share a set of highly conserved genes, mainly involved in virus replication. However, each gammaherpesvirus has also a set of unique genes, which are believed to be important for preventing premature death of latently infected cells and to regulate viral latency in the face of a perfectly normal immune system. Those unique genes are far less conserved among the gammaherpesviruses. Indeed, each of these viruses has evolved a unique set of such genes and a unique strategy for co-existence with its natural host.

In the context of pathogenesis, diseases due to gammaherpesvirus infections are mostly associated to latent forms of the causative viruses. Since the gammaherpesvirus genomes are episomal during latency and since latency is established in relatively short-living cells, there is a problem for these viruses to maintain a sufficient level of infected cells over time. EBV, the prototype of the lymphocryptoviruses, expresses the latency-associated nuclear antigen (EBNA-1 or LANA) throughout latency, which tethers the viral genome to the host chromosome to facilitate viral DNA replication synchronous with cellular DNA and to provide viral DNA to each daughter cell during mitosis. Other gammaherpesviruses, for example the rhadinovirus HHV-8, have a slightly different strategy. They provide newly infected cells from small areas of ongoing viral replication, which means that tissues infected with HHV-8 contain a mixture of latently and lytically infected cells. The strategy followed by the macaviruses, both in terms of viral gene expression throughout disease and maintenance of sufficient numbers of infected cells, is still a matter of debate [3,4,5,6,7,8] and have been addressed in the present PhD thesis.

2.2 Identification of MCF viruses

MCF has been defined as a clinico-pathological response to a variety of related viral agents [9]. Although the disease has apparently been recognized for over a hundred years, the first viral agent in association with it was detected only in the 1960ies [9,10,11]. Already in the early 20th century, MCF was described to be infectious and to be distributable among cattle by the transmission of blood [12]. In 1930, Götze et al published a collection of case studies identifying sheep as possible reservoir for the infectious agents in Europe [13]. Around the same time, a South African cattle disease termed *snotsiekte* and the European malignant catarrhal fever were declared to be one and the same disease.

In 1960, Plowright et al carried out large infection trials where blood, spleen and lymph node tissue from wildebeest was inoculated into cattle, which subsequently developed typical MCF [14]. These authors first isolated and identified a herpesvirus as the causative agent for the African variant of the disease and found that wildebeest serve as carrier for the virus and are the source for infection of cattle [10,11,14,15]. Later on, this virus became known as AlHV-1 [9,16]. Soon it became clear that more viruses, related but not identical to AlHV-1, were also able to cause MCF. Interestingly or unfortunately, depending on the point of view, it has not been possible to isolate the other candidate viruses in cell culture. It took until the 1990ies to unambiguously demonstrate by hybridization and PCR that the most widely distributed of those viruses, OvHV-2, also belonged to the herpesvirus family [17,18,19]. The sequence of this virus was only published in 2007, which then led to its final classification and to the establishment of the genus *macavirus* [1,20,21]. The present work focused on OvHV-2 and its pathogenesis. Therefore, it was vital to have the sequence of OvHV-2 available.

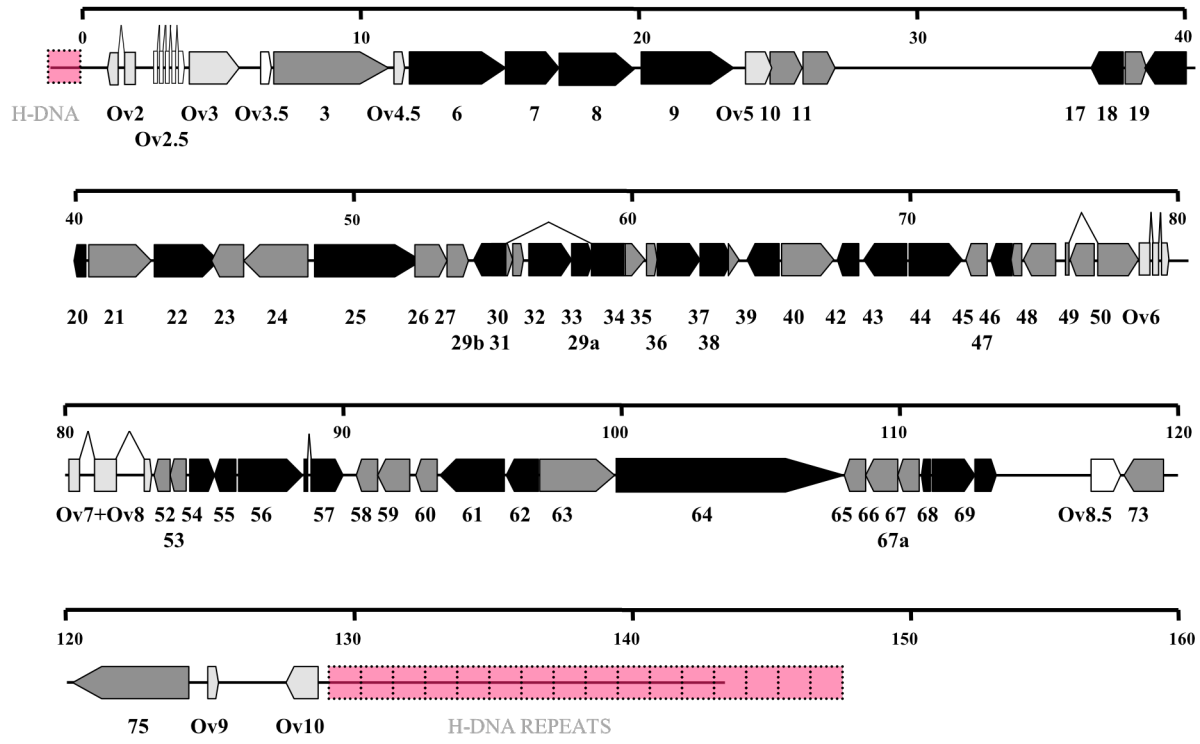
2.3 Genome

Heart et al published on the entire sequence of an European OvHV-2 isolate, derived from a lymphoblastoid cell line, which was obtained from a cow with MCF (BJ1035) [20]. The genome consists of about 140 kbp and 73 putative open reading frames (ORF). It is organized in conserved blocks, interspersed by variable regions (Figure 1). The content, position and orientation of the genes in the conserved blocks are conserved among the sequenced gammaherpesviruses. The conserved genes of OvHV-2 show large homology to genes of herpesvirus saimiri (SaHV-2), the prototype of the rhadinoviruses, and have therefore been assigned the number of the related SaHV-2 genes. Some of the OvHV-2 genes in the variable regions have homologues in AlHV-1. Ov2.5, Ov3.5, and Ov8.5 are unique to OvHV-2. The nomenclature indicates the position of the genes to neighboring unique genes of AlHV-1.

‘Ov’ and ‘A’ stand for unique genes of OvHV-2 and AlHV-1, respectively. The unique genes are believed to be vital in the context of pathogenesis *in vivo*. For example Ov2.5 encodes for a lymphocyte growth factor, a functional viral IL-10, which could explain unrestricted lymphocyte growth in animals with MCF as well as attenuation of the immune response in animal species that do not succumb to MCF [22]. Therefore, the potential activity of this gene was of interest in the context of the current work.

Simultaneously with the previous sequence, Taus et al published on the genomic sequences of an American OvHV-2 strain, which was derived from sheep [21]. Interestingly, the genome derived from sheep contained 74 ORFs instead of 73 as in the DNA retrieved from the cow. Due to a frame-shift, ORF40 and ORF41 appeared to represent two introns of a spliced gene in the first sequence but were identified as two separate ORFs in the sheep-derived OvHV-2 sequence. ORF40 / 41 encode for a helicase-primase. Similar findings have been made with genomes of other gammaherpesviruses [23,24]. Furthermore, there were differences between the European and the American strains in the amino acid (aa) sequences of ORF17, Ov3, Ov10 and ORF73. ORF17 was predicted to encode for a maturational protease. Ov3 showed similarities to a cellular semaphorine. The function of Ov10 remains unclear. The differences within ORF73 between the two sequences clustered in the N-terminus and the repeated residues in the central region of the gene. OvHV-2 ORF73 shows similarity to the latency-associated nuclear antigen (LANA) of HHV-8. Latency associated nuclear antigens are found in the genome of all gammaherpesviruses. They are essential for maintenance of the viral genome during latency and regulation of viral and cellular genes [25]. The N-terminus of LANA of HHV-8 binds to the core histones H2A/H2B to attach to chromosomes [26,27]. The C-terminus binds to the terminal repeat elements of the viral genome as well as to mitotic chromosomes, thereby tethering the viral genome to the host genome [28]. Both bindings are necessary for persistence and replication of the viral DNA. The central region was shown to reduce or even inhibit presentation of LANA epitopes to cytotoxic T-cells [29]. It is not yet known, whether or not the same functions apply for the OvHV-2 LANA orthologue. Therefore, the question about expression of ORF73 throughout MCF was of interest in the context of the present thesis.

Figure 1: Genome organization of OvHV-2.



2.4 OvHV-2 in sheep

As mentioned above, OvHV-2 is a sheep virus, circulating among all sheep breeds worldwide (reviewed by [30]). Although sheep represent the major reservoir for OvHV-2, the virus has also been detected in healthy goats. As a rule, lambs are born virus-free and are not infected with OvHV-2 prior to 2 – 3 months of age (reviewed by [30]). This is consistent with what has been observed in the context of AlHV-1, where wildebeest calves under the age of three months were shown to be the main source for infection of cattle [10,11,48,49]. Natural infection of sheep, independent of their age at the time of infection, proceeds without the slightest symptom of disease [31,32].

Viral DNA has been detected in sheep nasal secretions, suggesting that aerosols may play a role in transmission [33,34,35]. Apparently, shedding of high amounts of virus through nasal secretions occurs during primary infection but in a very limited time window of approximately 24 hours [35,36]. However, low levels of viral DNA can be found later on at intervals in nasal secretions of sheep. A rise in transcripts for structural viral proteins (ORF25, major capsid protein) and viral DNA copy numbers was detected up to 7 days post infection in lung cells of experimentally inoculated sheep [37]. This observation suggested that several cycles of replication may occur in the lung and that lung cells may be a source to produce infectious viral progeny. Importantly, nasal secretions from lytically infected sheep may be used as a source to transmit the infection experimentally [38]. Indeed, sheep, calves,

bisons, and rabbits have been successfully infected with OvHV-2 by intranasal nebulization of such nasal secretions [38,39,40,41]. MCF could be induced as a sequel to this way of transmission in calves, bisons, and rabbits. The incubation period (17 to 52 days) varied with the quantity of inoculated virus as determined by quantitative real-time PCR [38,40]. This is quite consistent with the natural incubation period of sheep, which varies between 6 and 26 weeks [35,42,43]. In search for tissues in latently infected sheep, which contained high amounts of viral DNA, the following ranking was determined [35]: the highest load of OvHV-2 DNA was detected in the small intestine, followed by lymphnodes, lung, and vesicular gland. The lowest amounts were detected in skeletal muscles, cerebrum, adrenal glands, cornea, and kidney. These observations supported the hypothesis of OvHV-2 transmission by the respiratory route. In addition, they suggested that, similar to HHV-8, veneric transmission also might play a role (reviewed by [44]). Indeed, high quantities of OvHV-2 DNA were detected in the ejaculates of OvHV-2-positive rams [35]. The same study also confirmed that OvHV-2 DNA was consistently detectable in the sheep white blood cells. However, the identity of the targeted blood cells in sheep remained undetermined. The present study, therefore, sheds light into this issue.

Interestingly, OvHV-2-positive sheep nasal secretions can be used to transmit the infection by nebulization but intravenous or intraperitoneal inoculation of the same materials does not lead to disease [37]. This suggests that OvHV-2 produced in sheep turbinate cells may not be able to directly target all types of cells susceptible to OvHV-2. Indeed, it has been shown in other gammaherpesviruses that different viruses can be produced, depending on the type of the infected cell. For example, a complex of three viral glycoproteins is essential for entry of EBV into B-cells: gp85, gp25, and gp42. Only three-part complexes can induce fusion with B-cells, since B-cell entry requires binding of gp42 to human leukocyte antigen (HLA) class II. In contrary, gp42 inhibits the infection of epithelial cells. EBV virions carry both the three-part and the two-part complexes. The ratio of the two influences the cell tropism of the virus. Virions made in B-cells lose some three-part complexes as a result of an intracellular interaction with HLA class II that targets the complexes to a degradative pathway. Thus B-cell derived virions rather infect epithelial cells. Epithelial cells are the site of an additional amplification. They are not HLA class II positive, thus virions derived from epithelia contain more three-part complexes and are able to infect B-cells of a new host [45,46]. The change in cell tropism may be the reason why so far it was not possible to establish productively OvHV-2-infected cell lines from PBMC. This issue will probably only become addressable for OvHV-2 once infectious clones of the virus similar to bacterial artificial chromosomes (BAC) for AlHV-1 can be made available [47].

OvHV-2 infection in sheep seems to be efficiently maintained by the immune system, which is in sharp contrast to what happens in animal species that fall to MCF. In sheep, at 7 days post nebulization with OvHV-2, an increase in numerous immune-associated cytokines, representative for activation of macrophages, dendritic cells, natural killer (NK) cells, T-helper cells type 1 (Th1) and Th2 cells as well as cytotoxic T-cells (Tc) can be observed, which coincides with a rapid decline of OvHV-2 DNA in the lung of affected animals [37]. Despite of the strong immune response, low levels of OvHV-2 DNA can be found regularly in nasal secretions of sheep.

2.5 Malignant Catarrhal Fever

MCF is a sporadic, mostly fatal disease of cattle and other cloven-hoofed animals, including deer, water buffalo, bison, and swine. A recent review describes the topic in more detail [30]. Briefly, the clinical symptoms are highly variable and the course can be peracute, subacute, or chronic. The first signs of MCF usually include dullness, inappetence, and elevated body

temperature. In addition, signs of mucosal inflammation begin to develop, including lachrymation, congestion of the conjunctiva and sclera, opacity of the cornea, salivation due to lesions in the oral cavity, increased nasal secretion as well as diarrhea [40,50,51,52,53,54,55,56]. Quite frequently, blood can be detected in the urine of affected animals. The case fatality is very high. With few exceptions, affected cattle die or have to be euthanized within approximately one week after onset of the disease. In deer (~48 hrs) and bison (~72 hrs), the disease takes even a more rapid fatal course [57]. Recently, there are increasing numbers of reports, emphasizing the recognition of chronic infections and subclinical courses in cattle, bison and deer [18,58,59,60,61,62,63].

Furthermore, there are seasonal peaks in the occurrence of MCF cases [43]. The frequency of MCF within an affected herd varies from single cases to the loss of a very high percentage of animals, depending mainly on the target species. Deer and American buffalo seem to be highly susceptible [54,55,64,65,66], compared to swine and cattle [38,67,68,69]. Still, high morbidity outbreaks of MCF in cattle and swine have also been reported [56,70,71,72].

Natural infection of rabbits has not been reported, but experimental infection leads to typical MCF symptoms such as fever, diarrhea, adipsia, anorexia and dullness [73]. Indeed, the rabbit model was used very successfully in the context of the present work.

2.5.1 Post mortem findings

Upon necropsy, widespread macroscopic lesions can be recognized, including reddening, erosion and ulceration in all sorts of mucous membranes, particularly in the respiratory tract, the esophagus, forestomacs, and intestines. Petechia and hemorrhages may also be noticed. The microscopic lesions include epithelial degeneration, vasculitis, hyperplasia and necrosis of lymphoid organs as well as interstitial infiltrations and accumulations of lymphoid cells in non-lymphoid tissues, particularly the brain [40,74,75,76]. Importantly, the post mortem findings in rabbits with MCF are quite similar to those of naturally affected animals [41,73].

Prior to the development and routine use of polymerase chain reaction (PCR)-based methods, histology was regarded as the gold standard for the confirmation of suspected MCF [19].

2.5.2 Immune responses during MCF and large granular lymphocytes

Although the immunological tools for bovines are under-developed compared to those of mice and men and tools for detecting antibodies against OvHV-2 are also scarce, it is very well known that cattle raise a strong immune response throughout MCF. A competitive ELISA, using a cross-reacting monoclonal antibody against an AlHV-1 protein, has been used to demonstrate development of serological responses against OvHV-2 in various animal species [19,49,62,63,66,77,78]. Most adult sheep worldwide react positive in this assay. In contrast, healthy cattle usually react negative, while a considerable fraction of cattle with MCF react positive [19,62,78].

Furthermore, MCF is characterized by T-cell hyperplasia involving proliferation and infiltration of lymphoid as well as non-lymphoid organs. Cytotoxic lymphocytes (CD8+) destroy the affected tissue, which results in extensive necrosis. T-helpercells (CD4+) are rather underrepresented. An interesting finding by Buxton et al. was that treatment of affected rabbits with Cyclosporin A reduced T-cell hyperplasia but did not prevent necrosis [5]. The low abundance of virus in affected tissue led to the hypothesis, that MCF as a disease may be caused by a misled activity of non-infected cytotoxic cells under the control of a small number of infected cells [5,6,7,79]. However, a recent study revealed a large number of viral DNA containing CD8+ cells in the vascular lesions of the brains of a cattle and a bison [8], giving raise to new discussions about the pathologic mechanisms leading to MCF.

Large granular lymphocytes (LGL) can be cultured from lymphnodes, thymus and spleen of diseased cattle, deer and rabbits [9,80]. CD4+, CD8+ as well as CD4 /CD8 double-negative T-cells have been grown in cell culture [7,81]. LGLs have an activated phenotype. Indeed, a constitutive activation of the *Lck* and *Fyn* tyrosine kinases was shown in LGLs derived from cattle with OvHV-2-associated MCF [79]. This finding reminds of similar observations from other gammaherpesviruses. For example, the latent membrane protein 2A (LMP2A) of EBV, the tyrosine kinase-interacting protein (Tip) of SaHV-2 as well as K15 of HHV-8 dysregulate lymphocyte signaling by interacting with Src family kinases such as *Lyn*, *Fyn*, *Csk*, and *Syk*, which are responsible for cell immortalization and activation in the absence of antigens [82,83,84,85]. Interestingly, all four above mentioned viral proteins have a transmembrane domain as well as *Src* homology domains.

OvHV-2-associated LGLs do not respond to Concavalin A stimulation, i.e. are already fully activated, are cytotoxic and MHC-unrestricted [9,81,86,87,88]. After adaptation, most LGL grow in absence of exogenous IL-2 and do not express the cytokine themselves either [81].

LGLs from cattle and rabbits with OvHV-2-associated MCF contain a mixture of circular and linear viral genomes, indicating that DNA replication goes on and that at least some lytic-cycle genes must be expressed. Indeed, such LGL can be used to transmit MCF by intravenous or intraperitoneal injection to experimental animals [69,80,89,90,91]. In contrast, the OvHV-2 genome is in a circular conformation in primary PBMC of either healthy sheep or cattle with MCF, indicating a latent state [3]. However, linear genomes prevail in PBMC of rabbits with MCF, which suggests that OvHV-2 is in a replicative state. Interestingly, in PBMC of rabbits with AlHV-1 associated MCF, the latent state is dominant [92]. Thus, there may be differences between various animal species and different MCF agents, all of which may complicate the overall view of MCF pathogenesis.

AlHV-1-associated LGL grow into characteristic large multinucleate foci that progress slowly [93]. Infectivity is strongly cell-associated and can only be further passaged by propagating intact cells. Following inoculation of these LGL into cattle or rabbits the animals succumb to MCF. This virus is, thus, referred to as the cell-associated virulent (CAV) form. After several passages, the cell cultures produce cell-free virus [93]. However, this form is not able any more to induce MCF in cattle or rabbits [94,95]. Therefore, it is termed cell-free attenuated (CFA). Attenuation is associated with genome rearrangements, i.e. translocation of DNA from the centre of the genome to areas next to or in between terminal repeat elements [95], thereby truncating and translocating genes. Genes involved in this attenuation are ORF50 and A6. ORF50 encodes for the replication and transcription activator (Rta), which is responsible for the induction of the lytic cycle of gammaherpesviruses [96].

In the present work, efforts were undertaken to identify contributing new insights into this complex picture. Since high concentrations of viral DNA have been detected in the lymphnodes of cattle with MCF and since lymphnodes play a major role in directing the adaptive immune response, we chose to analyze the cellular gene expression patterns in lymphnodes of cattle with or without MCF. Originally, it had been the plan to first FACS-sort those cells and only then analyze their gene expression pattern. However, sorted cells did not yield good enough RNA quality for that purpose. Therefore, we restricted ourselves to the analysis of the gene expression patterns of unsorted lymphnode cells.

2.6 Principles of activation and attenuation of antiviral immunity

As a general principle, incoming virus replicates first in tissue at the entry site. Most cells in these tissues, especially cells of the innate immune system, such as dendritic cells (DC), monocytes and macrophages and natural killer cells (NK) express sensors (Toll-like receptors, TLR) for general pathogen recognition (pathogen recognition patterns, PRP) in order to detect structures commonly associated to pathogens. Such structures, for example double stranded RNA, single stranded DNA, unmethylated CpG DNA, lipopolysaccharides, mannanns, etc., have been termed pathogen-associated molecular patterns (PAMPs)(reviewed by [97]). Depending on the activated TLR, different downstream signaling pathways are activated, leading to the activation of transcription factors, for example interferon regulatory factors and NF-kappaB (reviewed by [98]). Consequently, proinflammatory cytokines and chemokines are secreted, which further enhance the reaction of the innate immune system and activate the adaptive immune system.

Lymphocytes represent the most important effector cells of the adaptive immune system. They are subdivided into B- and T-lymphocytes, which circulate through the body, scanning for their cognate antigens. Upon meeting a suitable antigen, they become activated, expand in a clonal fashion and differentiate to acquire their essential function. The range of function may be divided into different effector functions and regulatory functions. Once the infection is resolved, the number of lymphocytes decreases again, and only memory cells persist.

Under normal conditions, at least three different signals are required to activate T-lymphocytes (reviewed by [99]). First, a T-cell has to establish binding of its T-cell receptor (TCR) to an antigen, which has to be bound to a major histocompatibility complex molecule (MHC). As a general rule, all nucleated cells express type 1 MHC molecules (MHC-I), whereas MHC-II molecules are typical for professional antigen presenting cells, such as DCs. Accessory molecules between the two interacting cells provide the second signal, for example binding of CD80/86 to CD28 and of CD40 to CD40L, respectively. The third signal is received through the interaction of the cells with inflammatory cytokines. After stimulation, at least three different types of activity can be associated to different types and subtypes of the lymphocytes.

T-helper cells. Naive CD4-positive (CD4+) T-lymphocytes (T-helper cells, Th) proliferate and differentiate into various effector subsets, which are characterized by the production of distinct cytokines, and which assign the cell's specific effector functions. At least three distinct Th subsets are known, Th1, Th2, and Th17 [100,101]. Th1 cells elicit delayed-type hypersensitivity responses, activate macrophages and are highly effective in clearing intracellular pathogens. Th2 cells are particularly important for the production of immunoglobulin E and eosinophilic inflammation and may suppress cell-mediated immunity (reviewed by [102]). Th17 cells are involved in tissue inflammation and autoimmunity. These cells produce IL-17A, IL-17F, tumor necrosis factor (TNF) and IL-6 (reviewed by [103]). IL-17 is a potent inflammatory cytokine [104]. During conventional infection with certain bacteria and fungi, IL-17 rapidly recruits neutrophils to clear the infection [105]. However, in autoimmunity, IL-17 may recruit effector T-cells into inflamed tissue [106].

Cytotoxic T-cells. Activated CD8-positive T-lymphocytes (CD8+) also undergo clonal expansion and preferentially differentiate into cytotoxic T-cells (Tc). Tc recognize infected cells through specific interaction with peptide-loaded MHC-I. As a consequence of this interaction, Tc will release cytotoxins, such as perforin and granulysin to form pores in the

membranes of the targeted cells and allow transfer of granzymes, which eventually kill the cells through the apoptotic pathway [107].

Antibody-producing cells. Naive B-cells have to bind antigen to their B-cell receptor and receive help from CD4+ cells in order to get activated. Consequently, they undergo clonal expansion and differentiate to produce and secrete antigen-specific antibodies.

Termination of the immune response. Once the danger is overcome by the specific immune system, it is very important to gain control over all the activities raised against invading pathogens. In this context, the development and function of regulatory T-cells (Treg) is essential (reviewed in [108,109]). At least three types of Treg are known. First, naturally occurring Treg, which express FoxP3, are characterized by the surface markers CD4+ and CD25+. CD25 is part of the interleukin 2 (IL-2) receptor. IL-2 is a key cytokine during inflammation and has multiple, sometimes opposing functions. It is a potent inducer of T-cell proliferation, effector T-cell differentiation, and of the development of cytotoxic T-cells. However, reduced IL-2 levels have been shown to be associated with autoimmune disease in mice and were described in a patient with IL-2 receptor deficiency, who presented with clinical symptoms comparable to MCF [110]. Indeed, IL-2 is important for the development, survival, and function of naturally occurring Treg and inhibits the development of inflammatory Th17 cells. Expression of the IL-2 receptor on T-lymphocytes is under strict control of the antigen-stimulated T-cell receptor, thereby determining the magnitude of the immune response (reviewed by [102,111]). Naturally occurring Treg exert their function both directly to activated Tc and indirectly by influencing antigen-presenting cells. Consequently, attenuation of the activated state of the Tc will result.

The second (Th3) and third (Tr1) types of Treg emerge only after induction. Th3 cells are positive for CD4 and TCR and secrete IL-10 as well as transforming growth factor beta (TGFβ), both of which contribute to attenuation of activated Tc. Tr1 cells are very similar but carry the CD8 marker instead of the CD4 marker. As yet, very little is known about the fates and functions of Treg in ruminants. However, it has been reported that in cattle gamma/delta T-cells, rather than CD4+/CD25+/FoxP3+ cells, are important for attenuating ongoing immune responses.

Throughout the present work, we identified the major blood cell types targeted by OvHV-2 in sheep, the natural host of OvHV-2. Furthermore, we addressed the cellular gene expression patterns that might affect and deviate the normal course of the antiviral immune response in cattle with MCF in comparison to healthy cattle.

2.7 Development of vaccines to prevent MCF

Since MCF is a viral disease, although caused by several distinct viruses, attempts have been undertaken to prevent the disease by immunization. Since the main agents, AlHV-1 and OvHV-2, are even serologically related [61,62,63,66,71,112,113,114], attempts were made to protect against OvHV-2-induced MCF by vaccinating with attenuated AlHV-1. However, this strategy was entirely unsuccessful [94,115]. In contrast, limited success has been achieved in the homologous AlHV-1-system.

Briefly, inactivated cell-free virulent AlHV-1 C500, a pathogenic strain isolated from an ox with MCF [94], induced incomplete protection against intravenously (i.v.) administered cell-free virus. Live cell-associated but not inactivated cell-associated virus protected rabbits against i.v. administered cell-associated virus [115]. In cattle, inactivated C500 given intramuscularly (i.m.) along with Freund's adjuvant protected most animals from WA-MCF, whereas intra-nasally applied virulent C500 provided only partial protection [116]. Interestingly, only low levels of neutralising antibodies emerged following the challenge infections, indicating that cellular immunity might play a more important role than humoral immunity for protecting cattle against WA-MCF [94].

In this context, it is interesting to note that attenuated strains of SaHV-2, which do not cause tumors, may protect susceptible animals from tumor development due to challenge infection with wild type strains [117]. Similarly, cellular immunity was shown to be of importance during HHV-8 and MHV-68 infection [118,119,120]. During seroconversion, HHV-8 specific CD8+ T-cells are detectable [121]. These are mainly directed against lytic cycle proteins. The immune responses observed after HHV-8 infection are rather strong and reach their peak 1-2 years after seroconversion. Cytotoxic T-lymphocyte responses to HHV-8 are considerably less frequent in KS patients than in asymptomatic HHV-8-seropositive subjects [122].

Although the issue of vaccination was not addressed in the present work, the results obtained here may be of importance for the future development of such. In the first place, surface antigens of the incoming virus at the portal of entry may be targeted by means of antibody-dependent immunity. As a second means, cytotoxic T-cells against viral antigens that are expressed intracellularly throughout the pathogenesis of MCF may help to avoid entering the detrimental pathway that is ensued in susceptible animals. Therefore, a major part of the present work concerned itself with viral gene expression in animals with MCF.

3 Hypotheses and aims of the thesis.

MCF is a complex viral disease with immunopathological aspects that are relevant only in certain animal species but not in others. With the causative agent(s) belonging to the gammaherpesviruses, it had to be envisaged that not only species but also the targeted cell type(s) might influence the behavior of the virus and, consequently, the outcome of the infection. Knowing about this complexity, we wanted to address questions that were on one hand crucial in the context and, on the other hand, could be solved within a reasonable time.

3.1 Aim 1. Which blood cells are targeted by OvHV-2 in sheep?

We hypothesized that one reason behind the species-specific behavior of OvHV-2 could be based on the possibility that different cell populations were targeted in either sheep or cattle, respectively. Since OvHV-2-positive LGL could be cultured from cattle with MCF and since these belonged to the T-cell family with CD4+ or CD8+ or CD4 /CD8 double-negative phenotypes [7,81], it seemed that these cells were the major target for OvHV-2 in animals with MCF. At the time when this thesis was initiated, no data were available for the corresponding situation in sheep. Therefore, it was our first aim to analyze over time the types of sheep blood cells targeted by OvHV-2. The results of these experiments are presented in publication 1 of this thesis.

3.2 Aim 2. Are structural viral antigens being produced in animals with MCF?

At the beginning of this study, knowledge on OvHV-2 had primarily been based on clinical, histopathological, immunological, and DNA-based evidence. Virus encoded proteins had not yet been detected. Furthermore, there were conflicting models from other gammaherpesviruses on the viral state throughout disease, one (EBV) requiring a latent viral infection as basis for disease progression, the other (HHV8) requiring a mixture of latently and lytically infected cells for the same purpose. Moreover, animals with MCF are not known to transmit OvHV-2 efficiently to other animals but infectivity had been associated to certain tissues of rabbits with MCF. Therefore, we hypothesized that tissues with infectivity might contain cells with OvHV-2 structural antigens. To test this hypothesis, we generated antisera against a predicted capsid protein as well as against a putative tegument protein of OvHV-2. These antisera were used for detection of structural viral antigens in rabbits with MCF. The results of these experiments are presented in publication 2 of this thesis.

3.3 Aim 3. Viral activity and host response throughout MCF

To better understand the driving forces behind MCF, it was of high interest to study the viral gene expression activity in a disease-relevant organ of animals with MCF. Similarly, it was attractive to simultaneously look for the host response in the same tissue. Since lymphnodes play an important role in the specific defense against viruses and since they were known to contain high loads of viral DNA, we hypothesized that important knowledge could be drawn from analyzing both the viral gene expression pattern in those lymphnodes and the relative host gene expression profile in comparison to the same tissue obtained from healthy animals. The results of these experiments are presented in publication 3 of this thesis.

4 Publications resulting from this thesis

4.1 Publication 1. Identification of peripheral blood mononuclear cells targeted by Ovine herpesvirus-2 in sheep

See reprint.

4.2 Publication 2. Ovine herpesvirus 2 structural proteins in epithelial cells and M-cells of the appendix in rabbits with malignant catarrhal fever

See reprint.

4.3 Publication 3. Malignant Catarrhal Fever of Cattle Is Associated with Low Abundance of IL-2 Transcript and a Predominantly Latent Profile of Ovine Herpesvirus 2 Gene Expression

See reprint.

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Identification of peripheral blood mononuclear cells targeted by Ovine herpesvirus-2 in sheep

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ABSTRACT

Sheep-associated malignant catarrhal fever (MCF), caused by Ovine herpesvirus 2 (OvHV-2), is usually a fatal disease of various ruminants and swine. In contrast, natural OvHV-2 infection in sheep, which are the main OvHV-2 reservoir, proceeds without any clinical symptoms. Since the range of targeted cells may be important for pathogenesis, we wanted to analyze the natural range of peripheral mononuclear blood cells (PBMC) targeted by OvHV-2. To this end, OvHV-2-free sheep were exposed to natural infection and blood samples were taken at intervals. Four different PBMC subpopulations were purified by fluorescence activated cell sorting (FACS) before being subjected to analysis for OvHV-2-DNA. After an incubation period of between 11 and 12 weeks, all exposed sheep became positive for OvHV-2. In most sheep, a first peak of OvHV-2-DNA was identified in the CD2 and CD4 double positive subpopulation. However, with time, the highest load of OvHV-2-DNA shifted to the CD2-positive and CD4-negative T-cells. Furthermore, low amounts of OvHV-2-DNA were occasionally detected also in the fractions that represented either CD14-positive monocytes or triple negative cells ($CD2^-/CD4^-/CD14^-$). We conclude from these experiments that OvHV-2 has a similar host cellular range in sheep and cattle, respectively. Our results may be relevant in the context of comparative analysis of OvHV-2 pathogenesis in animal species that are susceptible to MCF.

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1. Introduction

Malignant catarrhal fever (MCF) is a sporadic, usually fatal infectious disease of cattle, other ruminant species, and swine (Albini et al., 2003; Coulter et al., 2001; Hussy et al., 2002; Muller-Doblies et al., 2001a; Muller-Doblies et al., 2001b, 1998). There are two etiologically distinct forms of MCF: (i) a wildebeest-associated form, caused by Alcelaphine herpesvirus 1 (AlHV-1), and (ii) a sheep-associated form (SA-MCF), occurring worldwide and caused by Ovine herpesvirus 2 (OvHV-2). Based on their genomic sequences, both AlHV-1 and OvHV-2 belong to the Macaviruses within the subfamily *Gamma herpesvirinae* (Bridgen and Reid, 1991; Davison et al., 2009; Ensser et al., 1997; Hart et al., 2007; Taus et al., 2007). AlHV-1 can be propagated in cell culture (Castro et al., 1984; Plowright et al., 1960; Reid and Rowe, 1973). In contrast, there is no permissive cell culture system for OvHV-2, although OvHV-2-infected T-lymphocytes can be cultured from diseased animals (Coulter et al., 2001; Reid et al., 1989). Therefore, much less is known about OvHV-2 than about AlHV-1. Yet, due to the advent of molecular biological techniques and publication of the OvHV-2 sequences, some progress has been achieved in studying OvHV-2 virology and pathogenesis in recent years (Ackermann, 2006; Anderson et al., 2007; Cunha et al., 2008; Hart et al., 2007; Hussy et al., 2002; Jayawardane et al., 2008; Rosbottom et al., 2002; Taus et al., 2007; Thonur et al., 2006). Interestingly, this progress also led to the recognition of surprisingly high numbers of cattle and bison, which survive OvHV-2-infections with or without clinical signs of MCF (O'Toole et al., 1997; Powers et al., 2005; Yeslbag, 2007).

Sheep, the main reservoir host of OvHV-2, remain healthy upon natural infection with OvHV-2 (Ackermann, 2005, 2006; Hussy et al., 2002). Apparently, OvHV-2

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establishes latency in sheep peripheral blood mononuclear cells (PBMC), since the OvHV-2 genome is usually present in a circular conformation and the latency-associated ORF73 is transcribed, whereas transcripts corresponding to productive cycle genes ORF9 (DNA polymerase) and ORF50 (R-transactivator) are barely detected (Thonur et al., 2006). However, neither the range of PBMC that can be infected in sheep nor the dynamics of OvHV-2 infection in sheep has been subject to systematic analysis. Such knowledge may be valuable for better understanding the pathogenesis of clinical MCF in its differences to the subclinical pathogenesis of OvHV-2 in sheep. In order to progress in this direction, we took advantage of progeny from a specific OvHV-2-free flock of sheep (Hussy et al., 2001; Muller-Doblies et al., 2001b), which was divided into two groups. One group of lambs remained in isolation, whereas their siblings were introduced into a conventional flock of sheep with a history of OvHV-2 circulation. At intervals, blood was taken from both groups, the peripheral blood mononuclear cells (PBMC) were sorted, and the sorted cells were subjected to real-time PCR for OvHV-2-DNA in order to identify the major PBMC subpopulations that were targeted by OvHV-2. Using this approach, the CD2-positive lymphocytes were identified as the major acute and long-term target of OvHV-2 in sheep. Interestingly, the subpopulation carrying the highest load of viral DNA changed in the course of time after infection.

2. Material and methods

2.1. Animals

Six lambs in the age of 14–16 weeks were transferred from our specific pathogen free (SPF) OvHV-2-negative flock to an OvHV-2-positive flock. Five of the six animals were twins. Twins were divided to the two groups. Four of the six lambs of the positive and three of the negative flock were intact males. The original negative herd consisted of one ram and six adult females. The original OvHV-2-positive flock consisted of five adult females and three lambs, all of which were tested positive for OvHV-2. All animals were kept under conventional conditions. They were fed hay ad libitum and allowed daily access to pasture. During the experiment none of the animals developed clinical signs. Due to aggressive behavior all male lambs of the negative herd had to be castrated. One blood sample of each lamb was taken before the beginning of the trial to confirm that all lambs were OvHV-2-negative. Thereafter blood samples were taken in weekly intervals.

2.2. Sample preparation

10 ml EDTA blood was taken from each animal at weekly intervals. 40 ml ammonium chloride buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in bidest, pH 7.4) were added to lyse the erythrocytes. After 10 min, the samples were centrifuged and the supernatant was discarded. The pellet, containing the buffy coat cells, was then resuspended in PBS-A (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for fluorescence activated cell sorting (FACS) (see below).

2.3. Fluorescence activated cell sorting (FACS) and sorting strategy

All samples were kept on ice and shielded from light during the entire procedure. Buffy coat was washed three times with PBS-A and incubated for 22 min at 4 °C in the dark with PBS-A containing a mixture of diluted primary antibodies against CD2, CD4 and CD14 or CD8, respectively. After washing with PBS-A, the cells were incubated under the same conditions for 22 min with PBS-A containing the secondary antibodies (Table 1). Then, the cells were sorted in a FACSAria Flow Cytometer (Becton Dickinson) using the following strategy. Conditions for forward scatter (FSC) and side scatter (SSC, orthogonal light scatter) were adopted from the literature (Salzman et al., 1975; Thompson et al., 1985). First, the granulocytes and cell doublets were excluded by gating. In addition, we used previously described lymphocyte markers CD2 (Giegerich et al., 1989; Mackay et al., 1988), CD4 (Mackay et al., 1986; Maddox et al., 1985), CD8 (Ezaki et al., 1987; Maddox et al., 1985) and CD14 (Jacobsen et al., 1993) (Table 2), to separate CD2 and CD4 double positive cells, CD2-positive and CD4-negative cells as well as CD14 positive and CD2-negative cells from the "Rest", which comprised the cells negative for all three markers (CD2−/CD4−/CD14−) (Fig. 1). The sort was considered complete once 500,000 cells from each subpopulation had been accumulated.

To verify the accuracy of the sorting process a small aliquot of the sorted cells was reanalyzed using the same gates and settings as for the sorting process.

2.4. DNA extraction

Each 500,000 sorted cells were used as starting material for DNA extraction. DNA was prepared using the DNeasy Kit (Qiagen, Hombrechtikon, Switzerland) according to manufacturer's instructions. The columns containing the purified DNA were eluted with 200 μl of double distilled water. The amount of eluted DNA representing the DNA content of 25,000 cells was used in real-time PCR.

2.5. Real-time PCR

Real-time PCR for quantification of OvHV-2-DNA was done as described previously, with some modifications

Table 1
Parameters used to separate leukocyte populations.

Enrichment for	Sort according to cell size and granularity (FSC/SSC)[a]	CD2[b]	CD4[b]	CD14[b]
T-helper cells	+	Positive	Positive	Negative
Cytotoxic T-cells	+	Positive	Negative	Negative
Monocytes	+	Negative	Negative	Positive
"Rest"	+	Negative	Negative	Negative

[a] See also Fig. 1.
[b] Labeling with antibodies to be used as positive or negative sorting criteria.

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Table 2
Antibodies and working dilution used to stain cells for FACS.

	Antibody	Supplier	Clone	Isotype	Working dilution
1°	Mouse α-CD4[a]	VMRD	GC50A1	IgM	1:750
1°	Mouse α-CD8a[a]	VMRD	CACT80C	IgG1	1:750
1°	Mouse α-CD14[a]	VMRD	MM61A	IgG1	1:750
1°	Mouse α-CD2[b]	VMRD	36F-18	IgG2a	1:750
2°	Goat α Mouse IgG1-Cy5	Southern Biotech	1070-15		1:1000
2°	Goat α Mouse IgM-PE	Southern Biotech	1020-09		1:2000
2°	Goat α Mouse IgG2a-FITC	Southern Biotech	1080-02		1:1000

[a] Confirmed species activity against cattle, goat, sheep.
[b] Confirmed species activity against goat.

(Hussy et al., 2001). Briefly, 100 ng template (in 10 μl) were mixed with 25 μl Taqman Universal PCR Mix (Applied Biosystems) substituted with primers and probe. To control for unspecific inhibition, each sample was additionally tested at a dilution of 1:10 (10 ng DNA template). The test was considered valid when an appropriate Ct-difference was observed between the two dilutions. Furthermore, all samples were re-tested using a SYBR green-based qPCR targeting the Ov9 gene, essentially as described previously (Meier-Trummer et al., 2009).

For correlating the DNA input per sample to the corresponding cell numbers, the 18S Genomic Control

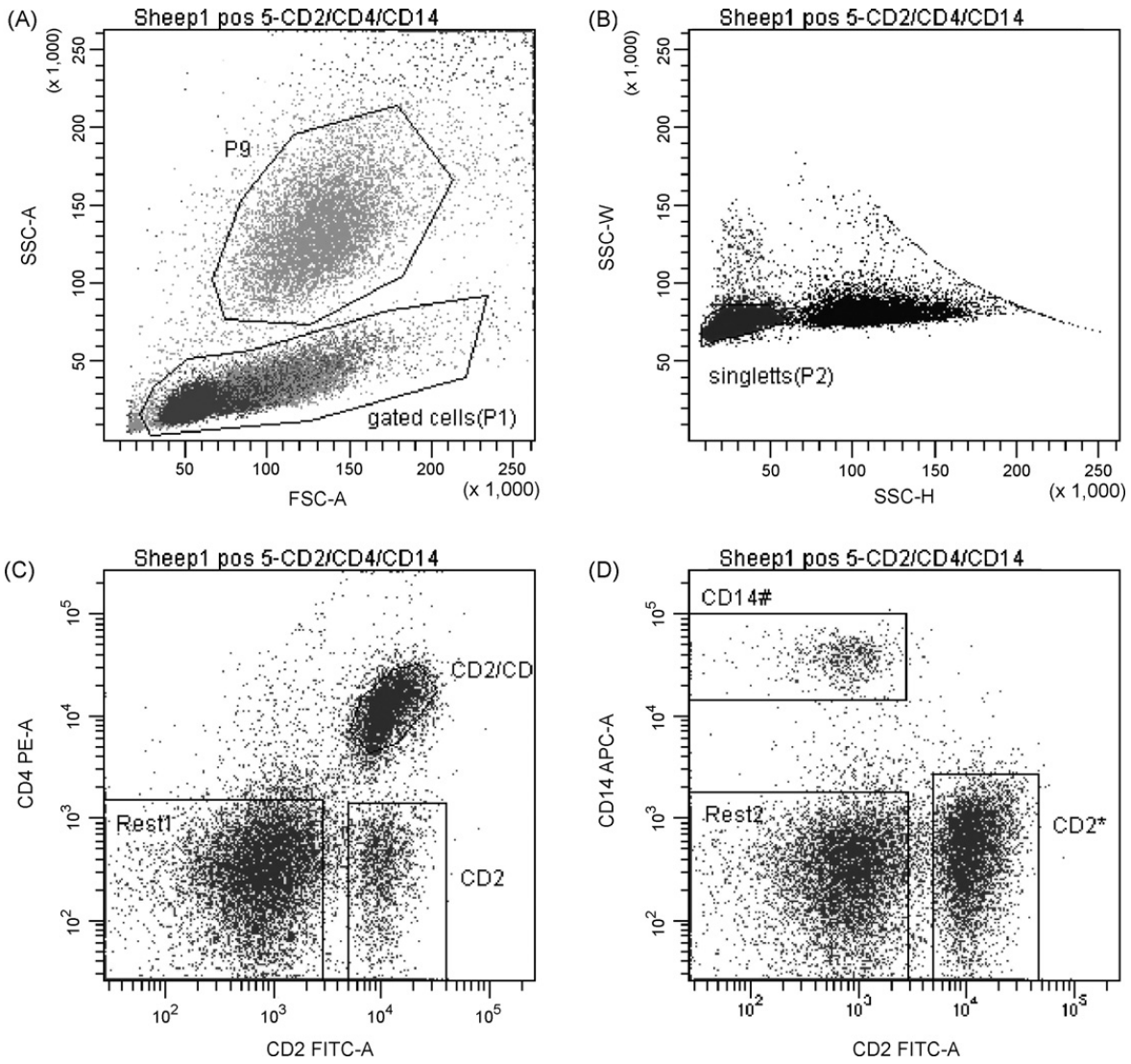

Fig. 1. Sorting strategy (sheep #1). Buffy coat cells were stained with antibodies against CD2 (FITC), CD4 (PE), and CD14 (APC) before FACS analysis and sorting. Panel (A) Cells were analyzed according to size (FSC, *x*-axis) and granularity (SSC, *y*-axis). P9 was discarded (granulocytes). Panel (B) Cells outside of P2 (doublets) were discarded. Panel (C) Cells included in P1 and P2 were sorted into CD2/CD4 double positive cells, CD2-positive and CD4-negative cells, and Rest 1. Panel (D) Similarly, P1 and P2 were sorted into CD14-positive and CD2-negative cells, CD2-positive and CD14-negative cells, and Rest 2. Rest1 and Rest 2 were combined to constitute "Rest".

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Table 3
Stages of OvHV-2-infection in naturally infected lambs.

Lamb	Conversion (stage 1)[a]	Peak in CD4+		Peak in CD8+	
		Week (stage 2)[b]	Copies[c]	Week (stage 3)[d]	Copies[e]
#1	12	13	35,120	14	7,104
#2	11	13	1,995	18	186,079
#4	12	13	349	19	12,158
#5	12	14	53,741	16	7,725
#7	12	15	691	18	704
#8	12	13	3,162	13	4,740
Siblings	n.d.	n.d.	0	n.d.	0

n.d.: not detected.
[a] Number indicates week post transfer of sheep to OvHV-2-positive flock, in which OvHV-2-DNA was first detected by PCR.
[b] Number indicates week, in which quantitative PCR revealed a peak value of viral DNA in CD2/CD4 double positive cells.
[c] Maximum number of viral DNA copies calculated to be present in CD2/CD4 double positive cells by quantitative PCR.
[d] Number indicates week, in which quantitative PCR revealed a peak value of viral DNA in CD2-positive/CD4-negative cells.
[e] Maximum number of viral DNA copies calculated to be present in CD2-positive/CD4-negative cells by quantitative PCR.

Kit – FAM-TAMRA by eurogentec was used according to the manufacturer's instructions (Seraign, Belgium) and in combination with Taqman Universal PCR Mix. These assays were run in duplicate, using two different dilutions of target DNA (10 and 1 ng). As above, the test was considered valid when an appropriate Ct-difference was observed.

All PCR reactions were optimized to reach a comparable high efficiency (Hussy et al., 2001; Meier-Trummer et al., 2009).

2.6. Quantification

Two standard curves were generated, one for quantification of viral DNA and the other for correlating those results with the number of cells in the sample. The following formula for the standard curves was used: $y = a \times x + b$, where y = Ct value of the sample, x = copy number of the sample, b = intercept and a = slope. The standard curve for quantification of viral DNA was based on a 10-fold dilution series of a plasmid comprising 467 nt of the PCR target sequence. To keep a constant overall DNA concentration in each sample, plasmid copies ranging from 10 to 10^6 molecules were added to a constant excess of DNA isolated from an OvHV-2-negative sheep.

For correlation with cell numbers, DNA was extracted from predetermined numbers of cells (10^3, 10^4, 10^5 or 10^6 of lymphocytes as measured in a Neubauer chamber). Then, the standard curve was generated by calculating these numbers against the Ct values obtained for 18S rRNA from each single DNA extraction. Mean values of duplicate samples differed by less than a Ct value of 0.4 and lay within a 75-percentile of close to 0.7Ct.

3. Results

3.1. Natural infection of lambs with OvHV-2

Six lambs in the age of 14–16 weeks were transferred from a controlled OvHV-2-negative flock to a known OvHV-2-positive flock. Siblings of the same lambs were kept in the negative flock. EDTA blood samples were taken from both groups of animals at weekly intervals to be tested for OvHV-2-DNA. The time point of the first appearance of OvHV-2-DNA was considered as a measure for the incubation time of the infection in each animal. The course of its quantity was used to describe the stage of the infection (Table 3).

A first screening revealed that all six exposed lambs turned positive for OvHV-2-DNA within a period of 11–12 weeks (Table 3). Specifically, lamb #2 converted in week 11 after exposure, whereas lambs #1, #4, #5, #7, and #8 converted in week 12. In contrast, their siblings (#3, #6, #9, #10, #11, #12) in the controlled OvHV-2-negative flock remained, as expected, negative for OvHV-2-DNA. All lambs, irrespective of exposure to OvHV-2, remained healthy throughout the experiment. These results suggested that natural infection by OvHV-2 had occurred in all lambs of interest, within a narrow time frame and without any negative consequences with regard to their health status.

3.2. Fluorescence activated sorting of individual white blood cell populations

Gamma herpesviruses are considered to have but a narrow host range, but only little is known yet about the natural cell range of OvHV-2. To address this issue, fluorescence activated cell sorting (FACS) was used. After collecting buffy coat cells, the granulocytes were excluded by gating and four fractions were enriched in order to be tested for presence or absence of OvHV-2-DNA, i.e. (i) CD2 and CD4 double positive cells, (ii) CD2-positive cells, which were negative for CD4, (iii) CD14-positive cells, which were CD2-negative, and (iv) the "Rest", which was negative for all markers used in the assay.

In Fig. 1, the results from a typical sorting experiment are shown.

Reanalysis with an anti-CD8 antibody was performed to test, whether or not the sorted CD2-positive and CD4-negative cells represented actually the CD8-positive lymphocyte population. Similarly, the CD4-positive population was re-tested for CD8-positive cells. The results of typical experiments are shown in (Fig. 2). Indeed, reanalysis confirmed that more than 97% of the sorted CD2-positive and CD4-negative cells proved to be CD8-

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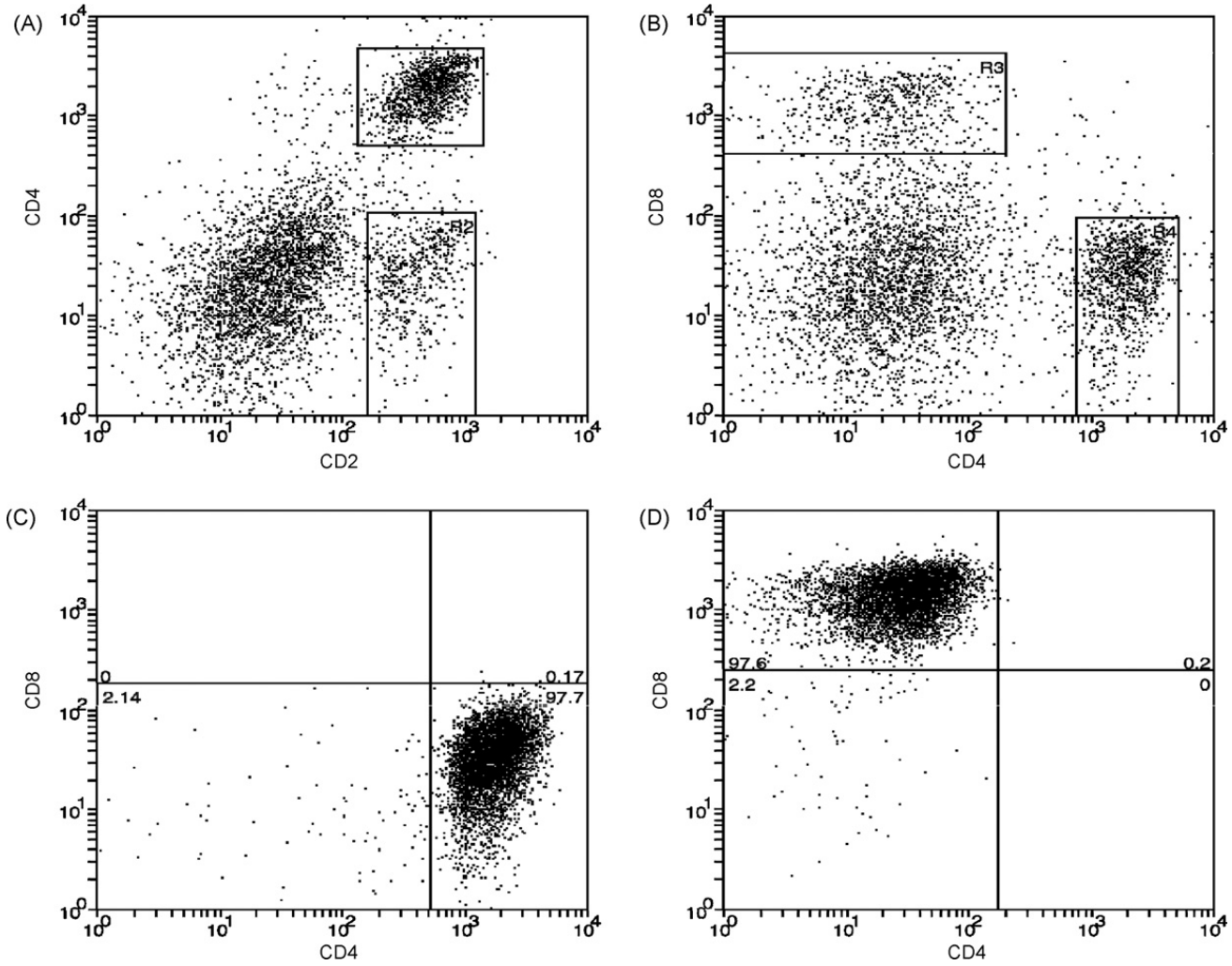

Fig. 2. Sorting and reanalysis of T-cells. Buffy coat cells were stained with CD2/FITC, CD4/PE, and CD8/Cy5 antibodies, respectively. Panel (A) shows a typical experiment for sorting CD2/CD4 double positive cells (R1) and CD2-positive/CD4-negative cells (R2). Panel (B) shows that CD8-positive cells (R3) were mainly CD4-negative, whereas CD4-positive cells (R4) were predominantly negative for CD8. Panel (C) Further analysis of R1 indicated that 97.7% of the CD2 and CD4 double positive cells were CD8-negative. Panel (D) Reanalysis of R2 showed that 97.6% of the CD2-positive and CD4-negative cells were CD8-positive.

positive. Similarly, more than 97% of the CD2-positive and CD8-negative cells were evaluated as CD4-positive. Indeed, reanalysis of all of the individual cell populations provided a mean purity of each sorted cell population ranging between 97 and 99%. Specifically, the mean purity of the sorted cells was 98.2% for CD4-positive T-cells, 97.6% for CD8-positive T-cells, 97.7% for CD14-positive cells, and 99.8% for cells comprised in the fraction that was negative for all markers used (“Rest”).

3.3. Determination of virally targeted peripheral blood mononuclear cell populations by quantitative real-time PCR

In order to determine whether or not, and if yes, to which extent a sorted white blood cell population was targeted by OvHV-2, DNA was extracted from 500,000 sorted cells of each addressed population and tested by quantitative PCR for the amount of viral DNA. As to be expected throughout natural courses of infection, both consistent and divergent observations were made. At specific time points, viral DNA was detected in all of the purified cell populations analyzed (Fig. 3). However, OvHV-2-DNA was only rarely detected in purified CD14-positive cells and in the Rest-fraction. The strongest signals were consistently detected either in CD2 and CD4 double positive cells or in CD2-positive and CD4-negative cells, which were considered to represent the CD8-positive CTLs. However, with time, the signal shifted more and more to the CD8-positive fraction, where the long-term reservoir seemed to be established. With regard to maximal values, three patterns could be discriminated (Table 3): (1) peak in CD4+ T-cells exceeding peak in CD8+ T-cells (animals #1 and #5); (2) peak in CD8+ T-cells exceeding peak in CD4+ T-cells (animals #2 and #4); (3) similar peaks in both CD4+ and CD8+ T-cells (animals #7 and #8). In five of six animals, peaking values in the CD4+ T-cells (weeks 13–15) were detected 1–6 weeks prior to the peak in CD8+ T-cells (weeks 13–19). Only lamb #8 showed the two peaks in the same week.

To investigate the possibility that the signal detected in those fractions was indeed attributable to the presence of virus in the sorted population, the probabilities for contamination were calculated based on the purity of the sorted cell fractions. Assuming constant ratios of cell populations in the starting materials, Table 4 shows that

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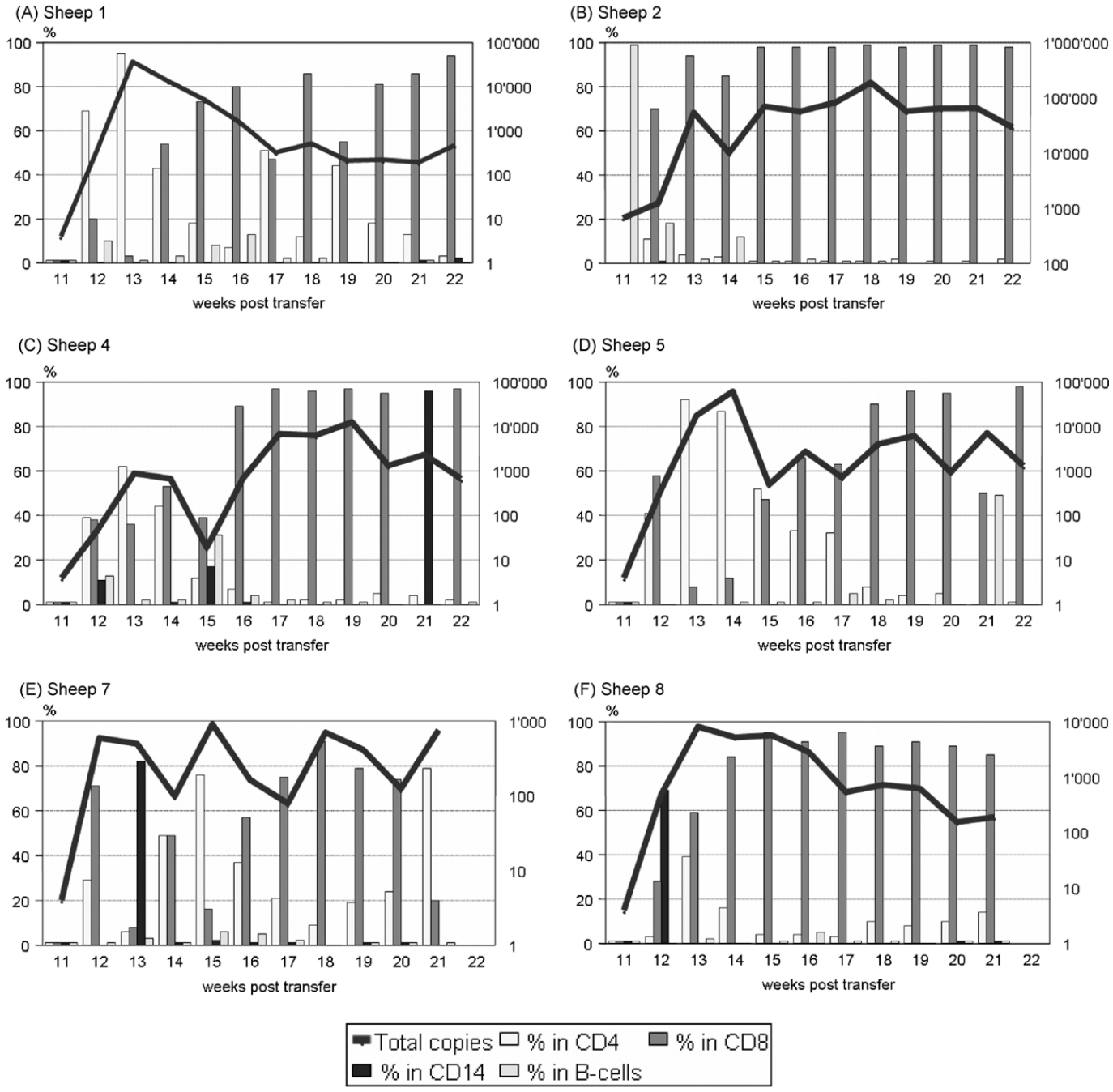

Fig. 3. Course of OvHV-2-DNA in sorted PBL fractions from naturally infected sheep. Buffy coat cells were sorted as described in the text and OvHV-2-DNA was measured by quantitative real-time PCR. Each panel (A–E) describes the result obtained from one individual lamb over time. The line in each panel describes the course of viral DNA copies determined at each time point. The bars indicate the %age of signal attributed to individual contributing cell populations. The *x*-axis indicates the time (weeks) after transfer of OvHV-2-negative lambs to a positive flock. The *Y*1-axis (%, left) applies to the bars. The *Y*2-axis (right, logarithmic) applies to the curve representing number of viral genomes. Note differences in the scale of the *Y*2-axis.

Table 4
Calculation of the number of potentially contaminating cells in the sorted subpopulations.

	CD2 + /CD4+	CD2 + /CD4-	CD14+	Rest
Relative amounts of white blood cell populations in adult sheep[a]	15.3 ± 3.0%	6.5 ± 0.5%	1-6%	47.5 ± 5.3%
Average purity[b]	0.982	0.976	0.978	0.998
Number of sorted cells[c]	491,000	488,000	489,000	499,000
Calculated number of potentially contaminating cells per 500′000	9,000	12,000	11,000	1,000

[a] Values according to literature (Tschuor et al., 2008; Woldehiwet and Sharma, 1990).
[b] Mean of 231 sorts, as determined by reanalysis.
[c] Average measured.

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the estimated number of potentially contaminating cells in each purified fraction ranged between 1000 and approximately 12,000 among close to 500,000 sorted cells. Based on those numbers, the probability was close to zero that contamination was the reason for OvHV-2-DNA detection in the purified monocyte population and the Rest cell population.

4. Discussion

Many Gamma herpesviruses have a seemingly narrow host range (Ackermann, 2004, 2006). However, OvHV-2 has a surprisingly broad natural host range as it may infect a wide range of animals, including sheep, goats, cattle, bison, deer, swine, and, at least experimentally, rabbits and hamsters (Ackermann, 2006; Albini et al., 2003; Audige et al., 2001; Berezowski et al., 2005; Buxton and Reid, 1980; Imai et al., 2001; Jacobsen et al., 2007; Li et al., 2008; Loken et al., 1998; O'Toole et al., 2007; Reid et al., 1979, 1986; Schultheiss et al., 2007). Interestingly, the fate of infected animals is vastly different, depending on the animal species affected. In sheep, OvHV-2 is virtually avirulent, whereas other animals, such as cattle, deer, swine, and bison, will develop malignant catarrhal fever (MCF) upon infection. One of the theories behind this discrepancy is the possibility that the virus targets different cells in different animal species. The other explanation may be that the nature of the infected host cell determines the pattern of viral gene expression, which, in turn, is an important determinant for its clinical consequences (Ackermann, 2006; Moore and Chang, 2003). Therefore, it is important to know the natural range of cells that are susceptible to the infection with OvHV-2.

For several reasons, this question is not trivial to address. First, the ways of natural transmission, the dose of infection, and the incubation times are still debated, although OvHV-2 has been successfully inoculated into sheep by means of nasal nebulization, using material derived from the respiratory tract of acutely infected sheep (Li et al., 2005; Taus et al., 2005). In order to avoid artificiality potentially caused by experimental infection, we chose to bring a group of OvHV-2-negative sheep into a flock of sheep with a history of OvHV-2 circulation, whereas their siblings were kept in the OvHV-2-negative flock. Under natural conditions, conventionally bred lambs are born virus-free but convert to OvHV-2-DNA-positive within a period of 2–6 months after birth (Hussy et al., 2001; Li et al., 1999; Muller-Doblies et al., 2001b). While their siblings remained free of OvHV-2, all six sheep transferred to the flock with OvHV-2 history turned OvHV-2-positive within a period of 11–12 weeks (Table 3), which is consistent with the literature (Hussy et al., 2002, 2001). Interestingly, the experimental incubation time is shorter, although dependent on the viral titer in the inoculum (Li et al., 2005).

Second, the sheep's immune system is not complete at the time of birth (Cole and Morris, 1973; Reynolds and Morris, 1983). In the first few days after birth, a decrease in white blood cell (WBC) counts is observed in lambs. After reaching a minimum WBC count of about 4×10^6/ml, the numbers gradually increase to reach maximal levels of approximately 9×10^6/ml at the age of 6 months. During this time, it is actually the number of lymphocytes, both T- and B-cells, that increases. Since natural infection of lambs with OvHV-2 essentially falls within this period of lymphocyte expansion, it has to be kept in mind that this process of immune maturation may also affect the outcome of the infection. Interestingly, we did not observe significant differences in the leukocyte numbers and subpopulations between OvHV-2-infected and uninfected lambs (data not shown).

Third, with regard to animal welfare restrictions, it was not possible to test all lambs on a daily basis. Therefore, we chose to test all animals at weekly intervals. Consequently, certain variations in the results had to be anticipated. Indeed, some of the inconsistencies experienced throughout our analyses may be attributed to this. For example, it may be that certain viral DNA peaks were not detected in some individuals due to those restrictions.

Despite of these limitations, it is now safe to state that OvHV-2 in sheep predominantly targeted the CD2-positive lymphocyte population (Fig. 3), which is most similar to its fate in animals susceptible to MCF (Anderson et al., 2007; Burrells and Reid, 1991; Thonur et al., 2006). According to our observations (Table 3), three stages of the infection were discriminated. After conversion to OvHV-2-DNA-positive (stage 1), in the majority of the sheep a first peak of viral DNA was recognized in the CD4-positive cell subpopulation, which probably represent the T-helper cells (stage 2). Later on, the signal for viral DNA was primarily detected in the CD2-positive and CD4-negative cells, which constitute probably mostly of CTLs (stage 3). However, it is important to state that OvHV-2-DNA was occasionally also detected in all of the other sorted populations, including the CD14-positive monocytes and the "Rest" population, which probably included B-lymphocytes as well as γδ T-cells. Although the purity of the sorted cell subpopulations may be slightly overestimated because we used the same criteria for sorting and reanalysis, the signals detected in the monocytes and the "Rest" population cannot be explained solely by contamination of the sorted cells with cells from other virus DNA-containing cell populations. However, the CD14-positive monocytes might also turn OvHV-2-positive upon phagocytosis of decaying infected T-lymphocytes. Caution is advised with regard to two unexpected shifts in the distribution of infected cell subpopulations in week 21 (Fig. 3): the sudden appearance of a high viral signal in the CD14 population of animal 4 and in the "Rest" population of animal 5 might be due to an error in labeling the tubes.

Despite of these concerns, it may be concluded from our observations and interpretations that the cellular host range of OvHV-2 in sheep is not tightly restricted to one single T-cell population. Indeed, the cells targeted by OvHV-2 in sheep seem to be the same as in cattle (Anderson et al., 2007; Burrells and Reid, 1991; Thonur et al., 2006). Therefore, we suggest that, rather than the mere types of targeted cells, a species-specific fine tuning of viral gene expression should be most important for the pathogenesis of MCF.

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Originally, it had been our plan to also address the state of viral infection in the sorted cells. Unfortunately, the quality of RNA extracted after sorting did not allow proceeding with this purpose (data not shown). In the absence of suitable antisera, it was also not possible to address the state of the infection in the individual cells by immunohistology. Therefore, it remains to be determined, which of those cells harbor replicating or latent virus, respectively. Interestingly, a recent model of AlHV-1-related MCF in rabbits suggests that AlHV-1, after replicating in unidentified cells, spreads eventually to CD8+ T-cells to establish a predominantly latent infection (Dewals et al., 2008). From this, the same authors insinuate that such a non-replicative infection may lead to proliferation and cytotoxic behavior of those cells. Similar observations have been reported for OvHV-2 in the context of MCF in cattle (Meier-Trummer et al., 2009).

Although many questions still remain to be answered, the present work provides, by describing the dynamics of OvHV-2 infection in sheep PBMC, some progress towards better understanding of OvHV-2 pathogenesis in sheep.

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Ovine herpesvirus 2 structural proteins in epithelial cells and M-cells of the appendix in rabbits with malignant catarrhal fever

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ABSTRACT

Sheep-associated malignant catarrhal fever (MCF), caused by Ovine herpesvirus 2 (OvHV-2), is a usually fatal disease of various ruminants and swine. A system for propagation of OvHV-2 *in vitro* has not yet been identified, although persistently infected cells have been derived from diseased animals and used to establish an animal model in rabbits. OvHV-2 structural proteins have not been detected in diseased animals and the pathogenesis of OvHV-2 infection is poorly understood.

Recently, the genomic sequence of OvHV-2 has been determined, which allowed to predict the amino acid sequences of putative OvHV-2 structural proteins. Based on those predictions, we have generated antisera against two putative structural proteins (ORF43 and ORF63) of OvHV-2 in order to detect sites of active virus replication in experimentally OvHV-2-infected rabbits with signs of MCF. Although histological lesions typical of MCF were detected in multiple tissues, those sera detected viral capsid and tegument antigens exclusively in the appendix but not in other tissues of rabbits with MCF. More specifically, those viral proteins were detected in epithelial cells as well as in M-cells. However, in situ hybridization revealed that ORF63 mRNA was present in epithelial cells of infected rabbits but not in M-cells. Our data suggest that active OvHV-2 replication takes place in certain tissues of animals with MCF and that M-cells may play a role in the pathogenesis of MCF.
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1. Introduction

Malignant catarrhal fever (MCF) is a sporadic, usually fatal infectious disease of cattle, other ruminant species, and swine (Albini et al., 2003; Coulter et al., 2001; Hussy et al., 2002; Muller-Doblies et al., 2001a,b, 1998). There are two etiologically distinct forms of MCF: (i) a wildebeest-associated form, caused by Alcelaphine herpesvirus 1 (AlHV-1), and (ii) a sheep-associated form (SA-MCF), occurring worldwide and caused by Ovine herpesvirus 2 (OvHV-2). Based on their genomic sequences, both AlHV-1 and OvHV-2 belong to the Rhadinoviruses within the subfamily Gammaherpesvirinae (Bridgen and Reid, 1991; Ensser et al., 1997; Hart et al., 2007; Taus et al., 2007). In contrast to AlHV-1, there is no permissive cell culture system for OvHV-2, although OvHV-2-infected T lymphocytes can be cultured from diseased animals (Coulter et al., 2001; Reid et al., 1989).

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Sheep, the reservoir host of OvHV-2, remain healthy upon natural infection with OvHV-2 (Ackermann, 2005, 2006; Hussy et al., 2002). Apparently, OvHV-2 establishes latency in sheep peripheral blood mononuclear cells, since the OvHV-2 genome is usually present in a circular conformation, while transcripts corresponding to productive cycle genes are barely detected (Thonur et al., 2006). In contrast, cultured T cells from diseased cattle contain a mixture of circular and linear genome configurations indicative of a mixture of latently and productively infected cells. Therefore, such cells can be used for the infection of experimental animals, such as rabbits and hamsters, which consequently develop an MCF-like disease (Anderson et al., 2007; Coulter et al., 2001; Reid et al., 1989; Thonur et al., 2006).

Between 5 and 23 days post-inoculation, infected rabbits develop MCF symptoms, which are most similar to those seen in cattle, i.e. fever, nasal and ocular discharge, conjunctivitis and diarrhea (Anderson et al., 2007; Buxton and Reid, 1980; Muller-Doblies et al., 2001a). Usually, they die within 2–3 days following the first febrile reaction. Sacrificed animals show hyperplasia of the lymphoid organs and lymphoid vasculitis in various organs (Anderson et al., 2007; Liggitt and DeMartini, 1980; Muller-Doblies et al., 2001a). OvHV-2 DNA can be detected and quantified in white blood cells as well as in proliferating lymphocytes within affected organs (Albini et al., 2003; Baxter et al., 1993; Hussy et al., 2002, 2001; Reid et al., 1986, 1989; Schock and Reid, 1996). Interestingly, transcripts for the OvHV-2 major capsid protein but not the corresponding proteins have recently been detected in several tissues from cattle and bisons as well as rabbits with MCF (Cunha et al., 2008; Gailbreath et al., 2008). The similarity of the clinical, pathological, histological, and molecular biological findings in rabbits and bovines with MCF suggest that a comparable pathogenesis leads to this disease, which makes the rabbit a valuable *in vivo* model for studying MCF. Since, under natural circumstances, MCF is hardly transmitted from one diseased animal to the other (cattle to cattle or rabbit to rabbit), the question arose, whether or not productive viral replication takes place at all throughout the course of the disease in those animals.

According to the recently determined genomic sequence of OvHV-2 (Hart et al., 2007; Taus et al., 2007), ORF43 encodes for an essential viral capsid protein, which has homologs in other herpesviruses, such as AlHV-1 (Ensser et al., 1997) and herpes simplex virus (HSV) (Newcomb et al., 2001). ORF63 matches to a tegument protein of AlHV-1, which is related to UL37 of HSV (Desai et al., 2001; Schmitz et al., 1995; Watanabe et al., 2000). To generate antisera for immunohistological detection of structural OvHV-2 antigens, parts of both ORFs were expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion proteins. The purified proteins were then used for the immunization of mice to generate specific antisera. Here we report on the detection of ORF43 and ORF63 proteins in the appendix of rabbits with MCF.

2. Methods

2.1. Infectious OvHV-2

Infectious OvHV-2 (Rosbottom et al., 2002; Swa et al., 2001) was derived from T cells isolated from a cow that subsequently succumbed to SA-MCF (BJ1035, from frozen stock) and from the first passage of the same isolate in rabbits.

2.2. PCR

The sequences of ORF43 and ORF63 are available from Genbank (AY366191 and AY366192). DNA sequences encoding for the ORF43 region H284-N435 and the ORF63 region N833-L944 were amplified from cosmid C33-63 using primers containing EcoRI sites for cloning of the products into the pGEX-6P-1 vector (Table 1).

The entire coding sequences of ORF43 and ORF63 were amplified from the same cosmid using primers containing BamHI (ORF43) and EcoRI sites (ORF63) for cloning into a HSV-1-based amplicon vector (pHSVPrPUC, provided by Dr. Howard Federoff).

For PCR, template (2 ng C33-63 or 7.5 ng pEGFP-N3) was mixed with 2 μl of each primer (10 μM), 3 μl *Pfu* buffer (Stratagene, Amsterdam, The Netherlands), 1 μl Pfu polymerase (2.5 U/μl) (Stratagene), 1 μl dNTP (10 mM, Amersham Pharmacia, Biotech, Dübendorf, Switzerland), in a final volume of 30 μl.

Following denaturation at 95 °C for 1 min, 35 cycles were completed at 95 °C for 1 min, 1 min at the temperature specified for each product in Table 1, and 74 °C for the time specified in Table 1.

2.3. GST-fusion proteins

The regions of choice were amplified by PCR and inserted into the EcoRI site of vector pGEX-6P-1 (Amersham). *E. coli*

Table 1
Primers for amplification of OvHV-2 DNA.

Oligonucleotide[a]	Sequence (5′–3′)[b]	Annealing (°C)/extension (min)	Product length
Fragment 43F	GATATCGAATTCCACCTAGATGAGTGTAGT[c]	50/1	478 bp
Fragment 43R	TAGAGAATTCCTAGTTAACCTGGTTGGAT[c]		
Fragment 63F	CTCCGAATTCAATCCCCAAATTTTCCTTGC[c]	52/1	356 bp
Fragment 63R	ACGCGAATTCTTACAGCTCCTGCGTAGGCT[c]		
43F	GTCGGATCCAGGATAAAGCTCTTAGGTATG[d]	60/2.5	1735 bp
43R	AGCGGATCCCGGGGCCCGCTACTCAGTCG[d]		
63F	CCTCTCTGGAATTCATGATGGAGAACAAGC[c]	55/4.5	2886 bp
63R	TGGAATTCGGCTGTGAGGCCATGGCGCTACG[c]		

[a] F: forward primer, R: reverse primer.
[b] Restriction sites underscored: [c]EcoRI, [d]BamHI.

BL21 (Amersham) were used for expression of protein according to the protocols of the manufacturer. After induction with IPTG, the bacteria were kept for 4 h at 30 °C before being harvested. The cell pellet was suspended in a mixture of 20 ml STE buffer (50 mM NaCl, 50 mM Tris base pH 8.0, 5 mM EDTA), supplemented with a protease-inhibitor cocktail (Sigma, Buchs, Switzerland) and lysozyme (100 mg/ml). The soluble lysate containing DTT and sarcosyl was sonicated before Triton X-100 (1% final concentration) and STE buffer were added to a final volume of 40 ml. This solution was mixed with glutathione sepharose beads (Amersham) in PBS-A (0.14 M NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 8.1 mM Na_2HPO_4) and incubated at 4 °C for 4 h before washing with PBS-A and elution in 0.5 ml 10 mM glutathione elution buffer. Similarly, GST was expressed from the original vector without fusion partner and the protein was purified as described above. The resulting protein concentrations were determined in a GeneQuant II Spectrophotometer (Amersham) at 280 nm, based on the predicted molecular weights and calculated molar extinction coefficients (Gill and von Hippel, 1989). The eluates were used for immunizations.

2.4. Production of labeled sense and antisense RNA

The same PCR products as used for cloning into GST-vectors were cloned into the EcoRI site of the transcription vector pSPT19 (Roche, Reinach, Switzerland). Prior to the transcription and labeling reaction using the DIG RNA labeling kit (Roche) according to the manufacturer's instructions, the plasmids were linearized with NaeI to produce antisense RNA (complementary to mRNA) and PvuII to produce sense RNA (corresponding to mRNA). Briefly, 2 μg of linearized DNA were incubated with 2 μl 10× NTP labeling mixture, 2 μl 10× transcription buffer, 1 μl RNase inhibitor and 2 μl RNA polymerase T7 or SP6, respectively, and incubated for 2 h at 37 °C. After DNA digestion and ethanol precipitation, the probe was resuspended in DEPC treated water and chemically reduced in size to about 200 nucleotides by incubating with 50 μl carbonate buffer (80 mM NaH_2CO_3, 120 mM Na_2CO_3, 60 °C, 25 min). After a further ethanol precipitation, the pellet was resuspended in 200 μl DEPC water.

2.5. Antisera against GST, GST-ORF43 protein, and GST-ORF63 protein

Standard methods were used to immunize C57BL/6 mice with either purified GST alone or with purified GST-fusion proteins. Preimmune sera were collected prior to the first vaccination and the mice received two immunizations at 3-week intervals. Mouse sera were collected 2 weeks after the second immunization and stored at −20 °C.

2.6. Monoclonal antibodies and conjugates

Mouse anti-Vimentin monoclonal antibody and anti-mouse-EnVision horseradish peroxidase conjugate as well as alkaline phosphatase-labeled anti-mouse polymer for double staining assays were obtained from DAKO (DAKO, Zug, Switzerland). Fluorescent Cy3 labeled conjugate was obtained from Stehelin AG (Basel, Switzerland).

2.7. Infection of rabbits

Six New Zealand white rabbits were infected intravenously with cell-associated OvHV-2. One rabbit (MP02/1092) received 10^8 infected cattle T cells (BJ1035). Five rabbits (MP02/1445; MP04/1045; MP04/1046; MP04/1189; MP04/1190) received 10^8 infected rabbit T cells taken from BJ1035 passage 1. Rabbits with clinical MCF were euthanized between day 9 and 16 post-inoculation, when rectal temperatures had risen to >40 °C for 2 days. Various tissues that are affected by OvHV-2 MCF were collected (appendix, lung, spleen, liver, mesenteric lymph nodes and kidney) and a portion of each tissue fixed in either 10% formal saline or zinc fixative (zinc salts consisting of 0.1 M Tris base, 0.05% Ca acetate, 0.5% zinc chloride and 0.5% zinc acetate). The same tissues were taken from uninfected control rabbits. According to histological analysis, the tissues from rabbits with MCF, but not those from healthy rabbits, showed typical lesions for example areas with hyperplasia of lymphoid cells within the appendix, mesenteric lymph node and spleen, separated from areas with ongoing necrosis/apoptosis. For more details, see elsewhere (Anderson et al., 2007).

2.8. Immunohistochemistry

Slides were deparaffinized in xylol and afterwards with a descending alcohol series and counter-stained with haemalaun. Endogenous peroxidase was blocked with water supplemented with 3% H_2O_2 and 0.2% NaN_3. To minimize unspecific reactions, the slides were incubated for 10 min with DAKO Protein Block Serum Free Solution (DAKO).

After washing with PBS, the slides were incubated over night with 100 μl of primary antiserum (1:500). The slides were washed and incubated for 30 min with peroxidase conjugate, anti-mouse-EnVision (DAKO, EnVision System with labeled polymer HRP anti-mouse, K4001). After washing, the slides were developed for 10–15 min with amino-ethyl carbazole (AEC) substrate (Stehelin).

For double staining, the same DAKO EnVision System with HRP anti-mouse was used in combination with DAKO EnVision Doublestain System (DAKO, K1395). First, the slides were treated as described above (primary staining). After development of the primary staining, the slides were incubated with DAKO Double Staining block solution and DAKO Protein Block Serum Free. The double staining block provides blocking of resident immunoglobulin Fc receptors, which are present due to the primary immunostaining. Then, mouse-anti-Vimentin monoclonal antibody was added for 30 min, followed by 60 min incubation with alkaline phosphatase-labeled anti-mouse polymer. The secondary staining was visualized using Fast red phosphatase chromogen solution (DAKO).

2.9. In situ hybridization

In situ hybridization was done using the DIG RNA labeling and detection kit (Roche) according to the

manufacturer's instructions. Briefly, slides containing tissue sections from OvHV-2-infected rabbits or uninfected controls were deparaffinized using xylol and descending ethanol series before being pretreated with DEPC water and PBS. After HCl treatment using 200 mM HCl for 20 min, the sections were permeabilized with 0.02 mg/ml RNase-free Proteinase K. Sections were prehybridized for 2 h at 50 °C with 0.25 mg/ml yeast RNA in 50 ml hybridization mix (50% deionized formamide, 4× SSC, 1× Denhard's solution). Hybridization was performed at 55 °C over night. Each section was overlaid with 40 μl hybridization mix containing 10% dextran sulfate, 0.6 μg/μl yeast RNA and 0.1 μg/μl of DIG-labeled antisense probe or 0.1 μg/μl of DIG-labeled sense probe.

To remove unbound RNA probe, the sections were washed first in RNase solution (500 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA, pH 8) containing 0.5 mg/ml RNase (DNase free) and 100,000 U/ml RNase T1 (Roche), then at 55 °C 2 times in 2× SSC and 2 times in 0.2 SSC, before being incubated for 2× 15 min at room temperature in blocking solution. For immunological detection, the sections were incubated for 1 h with 1500 mU/ml anti-digoxigenin-AP Fab fragments (Roche) in blocking solution. After two washings, the reaction was visualized using nitroblue tetrazolium solution (NBT) and 5-brom-4-chloro-3-indolyl-phosphate (X-phosphate) substrate. Finally, the slides were mounted with glycergel before analysis under the light microscope.

3. Results

3.1. Production and preliminary characterization of antisera against ORF43 and ORF63 proteins

In order to generate antisera against putative structural proteins of OvHV-2, antigenic parts of the ORF43 and ORF63 encoded proteins were expressed as GST-fusion proteins and purified. Similarly, GST without fusion partner was produced. These proteins were then used for immunization. The specificity of the newly generated antisera was first tested on Western immunoblots as well as in transiently with amplicon particles transduced cell cultures (Heister et al., 2004; Steinmann et al., 1998), which provided the desired products as well as appropriate controls. While the sera did not react with cells transduced with the unrelated construct, specific signals were observed in cells transduced with ORF43 amplicon and immune stained with the anti-ORF43 serum. Similarly, ORF63 protein was detected by the anti-ORF63 antiserum. A certain loss in sensitivity was observed in samples that had previously been fixed in formaldehyde and embedded in paraffin (data not shown). However, the produced antisera could be expected to specifically recognize their antigens, even in formalin-fixed and paraffin-embedded tissue.

3.2. Detection of OvHV-2 structural antigens in M-cells and epithelial cells in the appendix of infected rabbits

Previously, Rosbottom et al. (2002) reported that rabbit T cell lines supported productive OvHV-2 replication. In addition, infectious material had been harvested from gut-associated lymphoid tissue such as mesenteric lymph nodes and appendix (Schock and Reid, 1996). It was hypothesized that structural viral proteins might be detected in such tissues of infected rabbits. When sections from tissues (see Section 2) of OvHV-2-infected or non-infected rabbits were analyzed by immunohistology with mouse antisera against the putative tegument protein 63, specific labeling was detected exclusively in sections from the appendix of OvHV-2-infected animals. A strong signal was visible in the top layer of the dome epithelium of five of six infected (Fig. 1B) animals but not in uninfected animals (Fig. 1A). The signal was absent when preimmune sera (Fig. 1D) or sera against GST without fusion partner (not shown) were used. This staining pattern and the location of positive cells suggested that cells positive for OvHV-2 tegument protein 63 might be M-cells. Since vimentin is considered as a marker for rabbit M-cells (Gebert, 1995; Gebert et al., 1995), the same tissues from infected or uninfected rabbits were subjected to a consecutive staining with a monoclonal antibody against vimentin. While, tegument protein 63 was not detected in tissue from uninfected rabbits, M-cells were labeled in the same section (Fig. 1C). In contrast, double positive staining was detected in appendices from infected rabbits, mostly at the periphery of the domes (Fig. 1E and F). These observations suggested that structural protein of OvHV-2 were present in M-cells.

Similarly, epithelial cells but not goblet cells in the appendix of rabbits with MCF were shown to harbor OvHV-2 antigen by immunostaining with anti-ORF43 serum (Fig. 2). In contrast, tissues from uninfected rabbits revealed no immunostaining. The same infected rabbit that was negative for viral antigen in M-cells was also negative in epithelial cells.

These results demonstrated that in five of six MCF-affected rabbits, OvHV-2 structural antigens were present in epithelial cells as well as in M-cells of the appendix. This makes the link between cosmid cloned OvHV-2 nucleotide sequences, the encoded antigens, antisera produced to these, and viral native antigens *in vivo*. However, the nature of the infection in antigen-positive cells remained to be determined.

3.3. Detection of ORF63 mRNA in epithelial cells of the appendix from infected rabbits

Having detected viral proteins in cells from the appendix of OvHV-2-infected rabbits but not in other tissues that reacted positively for viral DNA by real-time PCR, it was of interest to further analyze the state of the infection not only in various tissues but also at the cell level. Preliminary testing by RT-PCR (not shown) indicated that ORF63 mRNA was detectable in extracts from appendix but not lung tissue of infected rabbits. Therefore, various tissues (mesenteric lymph nodes, lung, liver, kidney, spleen, and appendix) of rabbits with MCF were tested by in situ hybridization for ORF63 mRNA by using a probe anti-sense to the target. Representative results are shown in Fig. 3. ORF63 mRNA-expressing cells were detected exclusively with the anti-sense probe in the appendix of all six infected rabbits but not in other tissues.

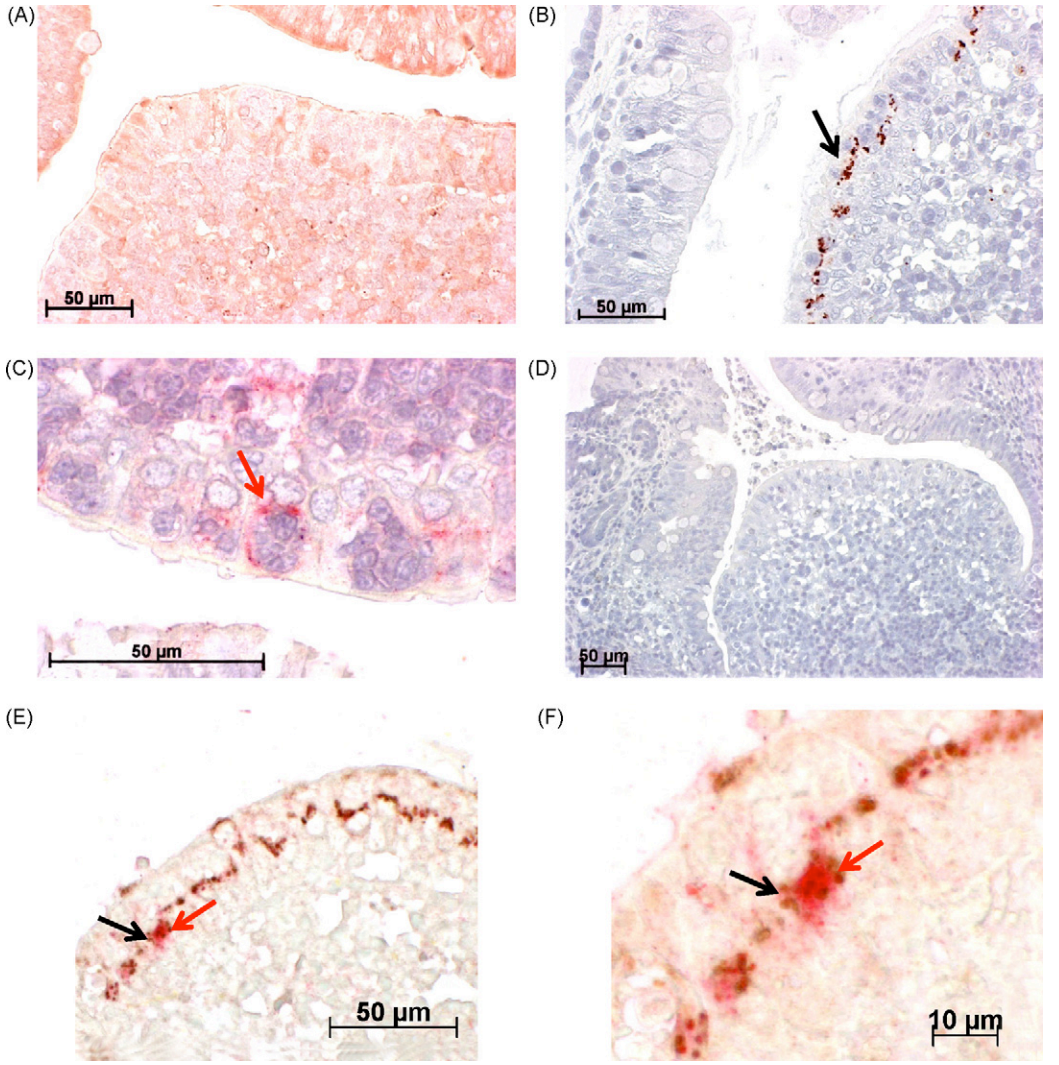

Fig. 1. ORF63 protein in M-cells from OvHV-2-infected rabbits. Histological sections from appendix of OvHV-2 infected (B and D–F) or uninfected (A and C) rabbits were subjected to immunohistology. (A) Uninfected rabbit, antiserum against tegument protein 63. (B) Infected rabbit, antiserum against ORF63 protein. (C) Uninfected rabbit, double staining with antiserum against ORF63 protein and antibody against vimentin. (D) Infected rabbit, preimmune serum. (E) Infected rabbit, double staining with antiserum against ORF63 protein and antibody against vimentin. (F) Same as (E) with higher magnification. Black arrow: viral antigen stained with anti-tegument protein 63 serum; red arrow: M-cells, stained with antibody against vimentin.

Specificity controls in this experiment included the use of a sense probe, which was unable to hybridize to ORF63 mRNA, omission of the probe, and hybridization on tissues from uninfected rabbits.

Most interestingly, ORF63 mRNA was detected solely in epithelial cells, but not in M-cells. The fact that the viral mRNA was detected also in appendix epithelial cells from the one infected rabbit that had not given positive signals by immunohistology suggested that absence of detectable viral mRNA in M-cells was not merely attributable to a lack of sensitivity in the technique. Rather, a biological explanation had to be considered.

4. Discussion

The main obstacle to a better understanding of the pathogenesis of sheep-associated malignant catarrhal fever is a lack of tools to study virus replication and other biological properties of ovine gammaherpesvirus type 2 (Ackermann, 2005). Production and characterization of specific antibodies against this virus has been difficult because OvHV-2 cannot be serially propagated as free virus in cell cultures. In the present study, we have overcome some of these shortcomings and provide insight into some novel aspects of OvHV-2 pathogenesis in rabbits with MCF.

The salient features of our work are as follows: (i) Antisera raised against two predicted structural proteins of OvHV-2 were able to establish the previously missing link between cloned DNA and viral antigens in tissues of animals with MCF. (ii) Both viral mRNA and the corresponding proteins were found in epithelial cells of the appendix of diseased rabbits. (iii) Viral protein but not the corresponding mRNA was found in M-cells of the same tissue. This supports

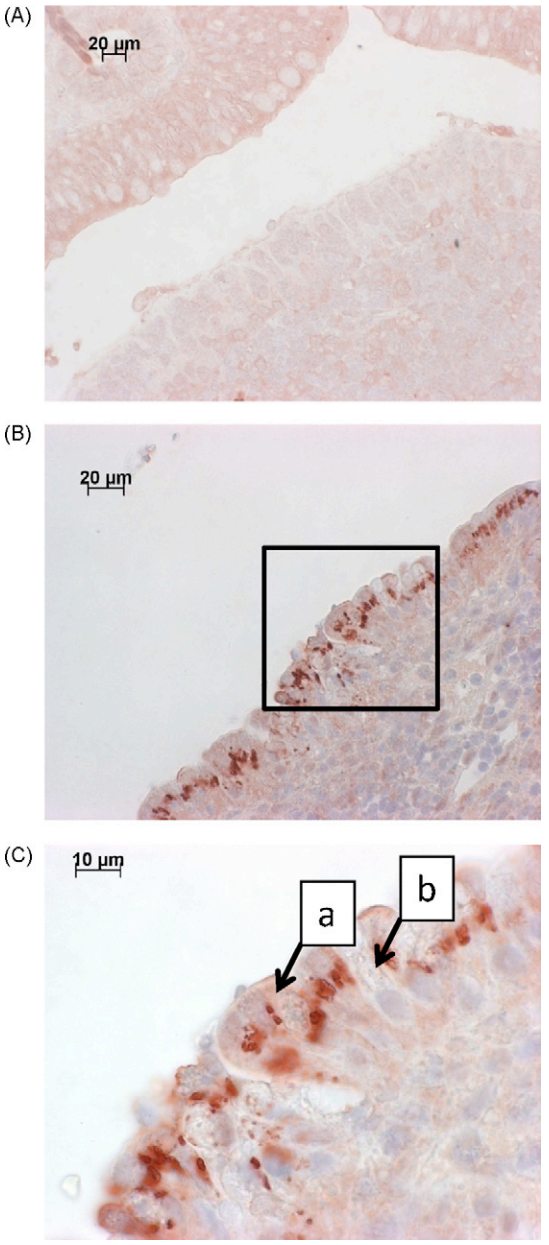

Fig. 2. ORF43 protein in epithelial cells from OvHV-2-infected rabbits. Gut tissues from the appendix were treated for immunohistology with anti-ORF43 serum. (A) Non-infected rabbit. (B) Section through epithelial cells in the crypt. (C) Section from (B) at higher magnification. Arrows: (a) viral antigen in epithelial cells; (b) goblet cell.

the hypothesis that M-cells may play an important role in gammaherpesvirus pathogenesis.

Based on the genomic nucleotide sequence of OvHV-2, two genes encoding putative structural proteins were selected for the present study, i.e. ORF43 and ORF63. According to its predicted amino acid sequence, the ORF43 protein is a homolog of herpes simplex virus UL6, which forms the portal for entry of the viral DNA into preformed capsids (Newcomb et al., 2001). Similarly, ORF63 has been predicted to encode for a tegument protein with homology to the UL37 protein of HSV (Watanabe et al., 2000). Therefore, detection of these two proteins can be considered as good indicators for ongoing viral replication in positive cells.

To generate antisera against those proteins, fragments thereof were expressed prokaryotically as GST-fusion proteins, which were purified and used to immunize mice. The newly generated antisera were carefully characterized (data not shown) before being used for immunohistology in various tissues of rabbits with or without MCF.

Similar to previous reports, typical histological signs of MCF were detected in appendix, lung, spleen, liver, and kidney of experimentally OvHV-2-infected rabbits (Anderson et al., 2007; Gailbreath et al., 2008). However, in our experiments, detection of OvHV-2 capsid and tegument proteins was restricted to certain cell types in the appendix, i.e. epithelial crypt cells and M-cells. Initially, the two cell types were discriminated on staining patterns and location of positive cells within the tissue. However, in a double staining experiment, the M-cells were also identified by using a monoclonal antibody against vimentin, which is considered a reliable marker for rabbit M-cells (Gebert, 1995; Gebert et al., 1995). When assayed by in situ hybridization, ORF63 mRNA was detected in epithelial cells but not M-cells from the appendix of rabbits with MCF. Since immunohistology does not correlate well with in situ hybridization, the epithelial cells and M-cells were discriminated in this instance solely based on their location.

On the one hand, the above observations are in good agreement with the presence of infectious cells in the appendix (Anderson et al., 2007, 2008; Buxton et al., 1984; Reid et al., 1986). On the other hand, others have reported on transcripts for the OvHV-2 major capsid protein in a variety of tissues from animals with MCF (Cunha et al., 2008; Gailbreath et al., 2008). Although the corresponding viral proteins were not assayed for in the latter case, this seeming contradiction provokes a debate on the accuracy and sensitivity of different detection methods or, alternatively, opens the possibility that biological differences may be responsible for the disagreement. On the one hand, it has to be considered that RT-PCR using tissue extracts as template is likely to be more sensitive than either in situ hybridization or immunohistology. On the other hand, the cell providing the corresponding signal can be identified by in situ techniques but not when using whole tissue extracts. Furthermore, it has to be kept in mind that we used a Scottish OvHV-2 isolate (Rosbottom et al., 2002) for inoculating the rabbits, whereas Gailbreath et al. (2008) used an American strain. Indeed, differences between the genomic sequences of European and American OvHV-2 strains, which may explain biological differences among the two, have been described (Hart et al., 2007; Taus et al., 2007). Finally, in our work the rabbits had been inoculated with cell-associated virus by the artificial, though widely accepted, intravenous route, whereas others have successfully made use of the intranasal inoculation of rabbits with cell-free virus (Gailbreath et al., 2008).

Despite of these reservations, detection of the OvHV-2 structural antigens in M-cells with the simultaneous

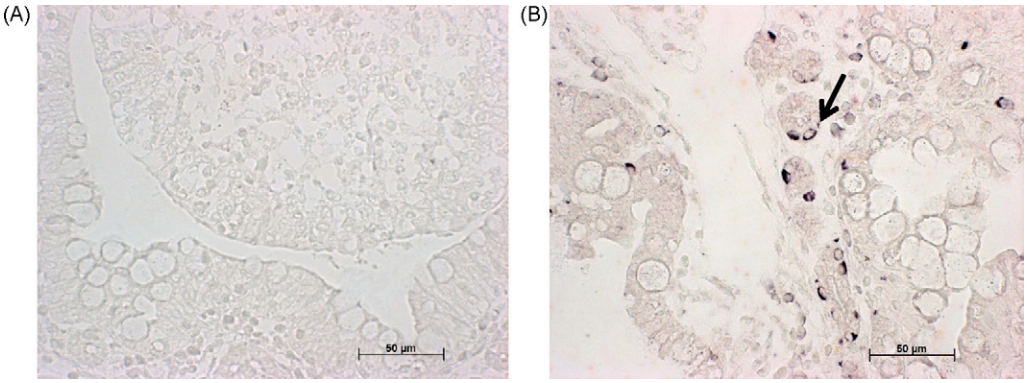

Fig. 3. In situ hybridization for ORF63 mRNA. Samples from the same infected rabbit are shown. (A) Sense RNA probe (same orientation as mRNA) and (B) antisense probe (complementary to mRNA). The arrow points to an epithelial crypt cell containing ORF63 mRNA.

failure to detect the corresponding mRNAs is notable. M-cells are specialized epithelial cells, located within mucosal surfaces lining the respiratory and intestinal tracts and capable of antigen presentation (Bockman and Cooper, 1973; Kabok et al., 1995; Owen et al., 1986; Sansonetti and Phalipon, 1999; Siebers and Finlay, 1996; Tizard, 2000). They take up antigens from the lumen in order to present them directly to lymphocytes. Interestingly, antigens that enter M-cells are likely to not be degraded but rather passed on to other cells or to the intercellular space. Indeed, soluble macromolecules, small particles, and even whole organisms are transported by M-cells. Therefore, some bacteria and viruses can use M-cells as a portal to enter the body (Fotopoulos et al., 2002; Fujimura et al., 2004; Siebers and Finlay, 1996). For example, a lymphotropic HIV-1 strain had been shown to cross M-cell monolayers before infecting underlying CD4(+) target cells (Fotopoulos et al., 2002).

The literature describes at least two possibilities for maintaining gammaherpesvirus infections in a specific organism. In the case of EBV, the EBV nuclear antigen 1 (EBNA-1) is able to efficiently tether the viral DNA to the host chromosome, both of which are duplicated throughout mitosis and provided to both daughter cells (Hu et al., 2002; Oehmig et al., 2004). Thus, EBV remains mostly latent in an infected organism. In contrast, the latency-associated nuclear antigen (LANA; homolog to EBNA-1) of human herpesvirus 8 (HHV-8) is much less effective in the same task. Therefore, maintenance of HHV-8 requires constantly ongoing viral replication, at least in some cells (Parravicini et al., 2000). It is possible that OvHV-2 in rabbits with MCF has a similar need for constant viral replication. Based on our results, we suggest a model according to which the epithelial cells in the appendix are productively infected, releasing infectious virus to the lumen. In a second step, M-cells would take up the virus from the lumen and transport it back into the organism for transferring it to lymphocytes, with which they interact closely. Such a circle would help to maintain the infection within the organism. Furthermore, this model could explain for the presence of structural viral antigens and the simultaneous lack of viral mRNA in M-cells.

An interesting observation came from one rabbit that fell sick with MCF following infection and showed ample amounts of ORF63 mRNA in its appendix tissue but failed to react positively with either one of the antisera against the predicted structural OvHV-2 proteins. Apart from purely technical explanations for this fact, it should be kept in mind that not all individual animals undergo the same course of disease following natural or experimental infection with OvHV-2 (Muller-Doblies et al., 2001b; Reid et al., 1986, 1989; Taus et al., 2005).

In conclusion, antisera raised against two predicted structural proteins of OvHV-2 were able to establish the previously missing link between cloned DNA and viral antigens in tissues of animals with MCF. Interestingly, both viral mRNA and the corresponding proteins were found in epithelial cells of the appendix of diseased rabbits, whereas viral protein but not the corresponding mRNA was found in M-cells of the same tissue. Our data corroborate the notion that M-cells may play a role as a possible route of entry for gammaherpesviruses (Faulkner et al., 2000).

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Malignant Catarrhal Fever of Cattle Is Associated with Low Abundance of IL-2 Transcript and a Predominantly Latent Profile of Ovine Herpesvirus 2 Gene Expression

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Abstract

Background: Malignant catarrhal fever (MCF) is a lethal disease of cattle, characterized by vasculitis, necrosis, and accumulation of activated, dysregulated cytotoxic lymphocytes in various tissues. Ovine gamma herpesvirus 2 (OvHV-2) is a causative agent of MCF, which may trigger the disease through immunopathogenic pathways. Lymphocytes are the main target of the virus. However, the pathogenic basis of the disease is still mysterious.

Methods/Findings: We hypothesized that the gene expression patterns of OvHV-2 and the relative abundances of host cell transcripts in lymphnodes may be used to identify pathways that help to explain the pathogenesis of MCF. Therefore, viral and host cell gene expression patterns in lymph nodes of animals with MCF and healthy controls were analyzed by microarray. Two regions on the viral genome were transcriptionally active, one encoding an orthologue to the latency-associated nuclear antigen (ORF73) of other gamma herpesviruses, the other with no predicted open reading frame. A vast number of transcripts related to inflammatory processes, lymphocyte activation, cell proliferation and apoptosis were detected at different abundances. However, the IL-2 transcript was eminent among the transcripts, which were, compared to healthy controls, less abundant in animals with MCF. The ratio between CD4- and CD8-positive T-lymphocytes was decreased in the lymphnodes of animals with MCF compared to healthy controls. In contrast, the same ratio was stable, when peripheral blood lymphocytes were analyzed.

Conclusions/Significance: The phenotype of mice with a deficient IL-2-system almost perfectly matches the clinical signs observed in cattle with MCF, which feature a significantly decreased IL-2 transcript abundance, compared to healthy cattle. This supports the hypothesis that immunopathogenic events are linked to the pathogenesis of MCF. IL-2-deficiency may play an important role in the process. Therefore, this work opens new avenues for research on MCF.

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Introduction

Malignant catarrhal fever (MCF) is a mysterious and lethal immunopathological disease of cattle and other cloven hoofed animals. Etiologically, MCF can be instigated by at least two distinct members of the Macavirus genus within the subfamily *gammaherpesvirinae*, i.e. alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2)[1]. Both viruses undergo subclinical infections in their natural reservoir hosts, whereas so-called indicator hosts, i.e. cattle, deer, bison, swine, succumb to MCF [2,3,4,5,6,7,8,9,10,11]. OvHV-2 is asymptomatically endemic worldwide in all breeds of sheep, giving cause to the occurrence of sheep-associated MCF (SA-MCF), wherever sheep and indicator hosts are kept in close vicinity [12,13,14]. In contrast, AlHV-1 is asymptomatically endemic in African ungulates, for example wildebeest, and MCF due to AlHV-1 is restricted to African countries or may occur on other continents upon contact of susceptible animals with infected Zoo animals [15].

The disease is characterized by the infiltration and accumulation of large numbers of CD8-lymphocytes, causing vasculitis and necrosis in a variety of tissues [10,16,17,18]. Various clinical patterns can be discriminated, i.e. a head-and-eye form, an intestinal form, and a cutaneous form [10,19,20]. The clinical findings may include combinations of ocular and nasal discharge, opacity of the cornea that may lead to blindness, diarrhea, haematuria, erosions of the muzzle, lymphnode swelling and eventually erosions on the skin [10,16,17]. However, the symptoms are often not clearly attributable to one of the clinical patterns, e.g. diarrhea occurs in nearly all affected animals.

While AlHV-1 has been isolated in the 1960ies and can be serially propagated in cell cultures, there is no suitable monolayer cell culture system to serially propagate OvHV-2 [2,3]. Therefore,

it has been difficult to study OvHV-2 and its underlying pathogenesis in either sheep or cattle. However, much progress has been achieved in recent years due to advances in molecular biological techniques. Initially, it was the detection and analysis of herpesvirus-like DNA in tissues from animals with MCF, which allowed consecutive establishment and improvement of various PCR detection and quantification methods for OvHV-2 [6,21,22,23]. These technical developments allowed for studies on virological, epizootological, and pathogenetical aspects of OvHV-2 in various animal species and also for the establishment of a very useful rabbit model of the disease [24,25,26].

OvHV-2 exhibits typical features of a gamma herpesvirus, which has been confirmed through the recent completion of its genomic DNA analysis [27]. OvHV-2 has a double stranded DNA genome, which can be divided into a unique long fragment of approximately 130 kbp and multiple copies of approximately 4 kbp terminal repeat elements. The genome encodes for at least 73 open reading frames (ORFs), 62 of which show homology to known gamma herpesvirus genes. Among others, a gene encoding for a latency-associated nuclear antigen (LANA), a key feature of gamma herpesviruses, was predicted in ORF73. The other genes are either shared with AlHV-1 or unique to OvHV-2 (Table 1). Among this second set of genes, there are several candidates that could provide explanations for the disease phenotype, which includes uncontrolled multiplication of lymphocytes in various tissues. For example, a spliced homolog of cellular interleukin 10 (vIL-10, Ov2.5) has been described, which may serve as a growth factor for the host's lymphocytes and which also may act differently, depending on the animal species infected. Furthermore, two homologs to Bcl-2 (Ov4.5 and Ov9) have been identified. These might contribute to the protection of infected cells against intrinsic or extrinsic apoptosis, which is induced in the course of a normal immune reaction to prevent uncontrolled multiplication of activated lymphocytes (reviewed by [28]).

The major sites harboring high amounts of viral DNA in the course of MCF comprise blood lymphocytes and organs of the immune system, including spleen and lymphnodes. However, the presence of viral DNA in each single dysregulated cell, which contributes to the disease picture, has been a matter of debate. Some authors believe that only a fraction of lymphocytes is infected, whereas others argue that the frequency of virus positive cells *in vivo* is being underestimated due to the lack of sensitive methods for detection [6,24,25,29,30].

Table 1. Unique OvHV-2 genes.

Unique gene	Possible function
Ov2	Regulation of transcription; leucine zipper protein
Ov2.5	Viral IL-10 (vIL-10)
Ov3	Intracellular signaling; semaphorin family
Ov3.5	No prediction, unknown
Ov4.5	Cell death regulator; Bcl-2 family
Ov5	Intracellular signaling; G-protein coupled receptor
Ov6	Viral transactivator; similarity to Zta of EBV
Ov7	Glycoprotein
Ov8	Glycoprotein
Ov9	Cell death regulator; Bcl-2 family
Ov10	Transcriptional regulation; nuclear localization signal

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Based on these arguments, we set out to test the following two hypotheses:

(i) Development of MCF is associated with increased survival and multiplication of latently infected lymphocytes, which are protected from apoptosis through functions of a specific set of viral proteins, including Ov2.5, Ov4.5, and Ov9 (Table 1). The expression of the corresponding viral genes in diseased animals can be measured by a viral microarray. Survival levels of infected cells could be increased through direct interaction of viral proteins with cellular proteins, which regulate apoptosis in activated lymphocytes. In this case, the gene expression patterns of the infected cells would not necessarily be different from those of uninfected cells. In the same assay, a predominantly lytic type of viral gene expression was expected to be recognizable.

(ii) Alternatively, viral proteins or micro RNAs could influence the cellular gene expression patterns, which can be recognized through a microarray analysis of cellular gene expression. In this case, the pathogenesis of MCF could also be based on a dysfunctional interplay between the cells involved in immune functions. In such a model, only a fraction of relevant cells needs to be infected to allow for this type of pathogenesis. Furthermore, the pattern of viral gene expression may be distinct from that proposed in the first hypothesis.

While a lytic type of virus infection would be difficult to explain, in both alternative cases, the normal pathways to restrict multiplication of activated lymphocytes by induction of apoptosis would be disturbed, which could result in dysregulated multiplication of lymphocytes as a basis for the disease phenotype.

To test these hypotheses, we generated a microarray for the semi-quantitative detection of viral transcripts. Labeled cRNAs were tested on the viral microarray as well as on a cattle microarray comprising the relevant genes for analyzing the general features of the host's status of immune response. Important findings were corroborated by alternative methods. We found that, indeed, MCF was associated predominantly with a latent type of viral gene expression and, furthermore, we may have detected an important clue to understand and, possibly, treat MCF in the future.

Results

Lymphnodes are one of the main sites for diagnosis of MCF and lymphocytes are the main carriers of OvHV-2 DNA in cattle with MCF. In order to get insight into the pathogenesis of this disease, an effort was undertaken to analyze the cellular and viral transcription profiles in such lymphnodes and to compare the cellular transcription profiles of animals with MCF to those of uninfected animals. For this purpose RNA was extracted from OvHV-2-positive lymphnodes of cattle with MCF as well as from OvHV-2-negative, healthy control animals. Consecutively, Cyanin- and biotin-labeled cRNA was produced for use in microarray analysis and standardized as described in Materials and Methods. Presence and quantity of selected viral RNAs was additionally assayed by qRT-PCR.

Analysis of viral transcripts

Microarray. In a first set of experiments, the Cy3- and Cy5-labeled cRNA was used for hybridization with an array, which represented the entire OvHV-2 genome. Specifically, 8730 60mer oligonucleotides were used as viral targets. These oligonucleotides

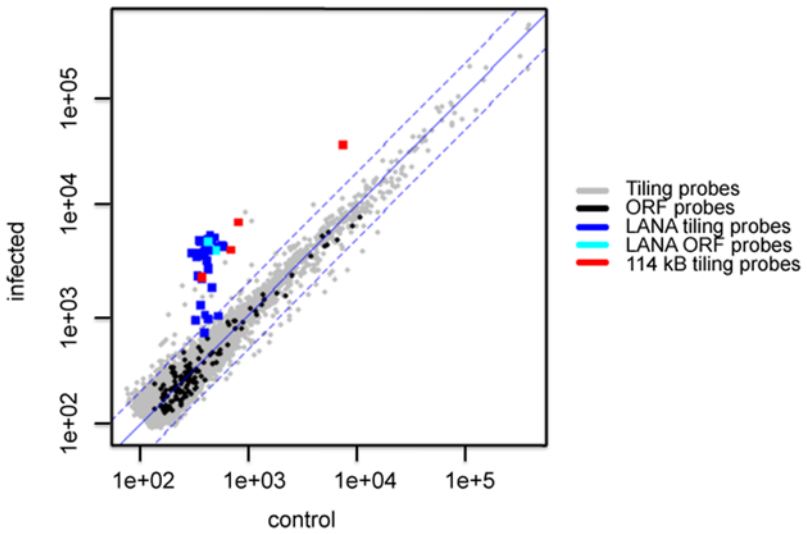

Figure 1. Hybridization signals. Comparison of the hybridization signals of infected (MCF-diseased) and control animals on the viral microarray. The tiling and ORF specific probes are plotted in gray and black. We have highlighted the probes matching the LANA homologue (blue/cyan) and 4 consecutive tiling probes targeting the region 115250 bp on the forward strand (red).
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represented both strands of the viral genome twice (tiling probes). To generate a second type of screening for transcriptional activity, two additional oligonucleotides per each predicted viral open reading frame (ORF) were used as targets, which had been selected for optimal hybridization under the conditions used (ORF probes). The array was hybridized and analyzed as described in Materials and Methods. The resulting data were deposited in the GEO database (GSE13853). Figure 1 compares the hybridization signals obtained from cells of infected and control animals. The points represent the expression signal of the probes targeting the viral genome. The majority of the probes (gray and black points) did not show any differential signal. Only two regions on the virus genome showed transcriptional activity. The first one covered ORF73, which is located on the reverse strand spanning the region 119046 bp to 120533 bp and represents a LANA orthologue. In this region 31 consecutive tiling probes showed differential signals in the infected animals (see Figure 2). The same was also observed by the two probes that were optimized to target the ORF73 (cyan dots in Figure 1). Thus, transcription from ORF73 was detected using two alternative methods.

The second region for viral transcriptional activity spanned nucleotides 115184 to 115364 bp on the forward strand of the viral genome and was represented by four consecutive tiling probes. This region corresponds to an intergenic region, located between ORF69 and ORF Ov8. This observation was unexpected and invites for further analysis.

qRT-PCR. The present microarray was designed only to provide relative information towards viral transcriptional activity throughout MCF. In order to validate the presence of the transcripts predicted by the viral microarray and to explore the sensitivity of the present assays, a number of quantitative reverse transcription real-time PCR (qRT-PCR) assays were established (see Materials and Methods) and used for detection and quantitation of selected viral RNAs in lymphnodes of cattle with MCF. The results are summarized in Table 2. Briefly, the sensitivity of the assay to detect ORF25 template was comparable to that for detecting the intergenic target, whereas the tests to detect ORF73 and Ov9 were more sensitive. Under these conditions, the ORF25 transcript, encoding the major capsid protein of OvHV-2, remained undetectable in our materials,

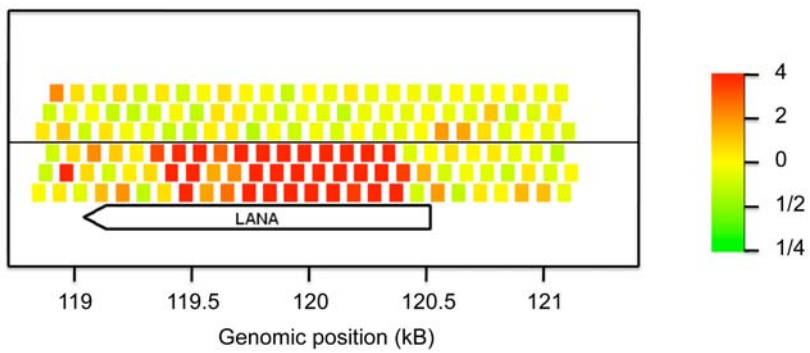

Figure 2. OvHV-2 gene expression. Expression pattern in the region 119 kB to 121 kB of the virus. The plot shows the expression changes measured by the probes tiled across the genome and the predicted location of the LANA gene. Probes above (below) the dividing line match the forward (reverse) strand. Significant consistent induction is measured by the probes matching the reverse strand at the locus of ORF73 (LANA orthologue).
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Table 2. qRT-PCR for selected viral RNAs in lymphnodes.

Target	DNA[a]	Melting peak[b]	RNA (MCF)[c]	RNA (healthy)[d]
ORF25	100–1000	n.a.	Not detected	Not detected
ORF73	>10	81°C	100–1.000	Not detected
Ov9	>10	81°C	Not detected	Not detected
Intergenic	>100	83°C	500–10.000	Not detected

[a]detection limit (number of template copies) using DNA template.
[b]determined following qPCR using SYBR-green technology; not applicable (n.a.) to conventional PCR, which was detected by EtBR staining after agarose gel electrophoresis of the PCR product.
[c]25 ng RNA template (from lymphnodes of animals with MCF) before reverse transcription. Numbers in the column refer to copy number detected.
[d]25 ng RNA template (from lymphnodes of healthy animals) before reverse transcription.
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whereas the intergenic transcript was present at high numbers. Similarly, between 100 and 1.000 copies of the ORF73 transcript were detected using the same amount of template that did not reveal any transcriptional activity over Ov9. Calculating conservatively, the microarray used throughout our experiments had a detection limit of less than 100 copies per assay.

From all of the above experiments we conclude that no transcripts corresponding to structural viral proteins were detected. These results supported the notion that MCF was associated with a predominantly latent OvHV-2 infection. However, they also clearly, contradict hypothesis 1, which predicted that several viral genes would be expressed, which are unique to OvHV-2 or shared with AlHV-1 (see Table 1). Indeed, no significant transcriptional activity in any of those genes was detected under the present experimental conditions.

Analysis of host transcripts in animals with and without MCF

The biotin-labeled cRNAs were also used for hybridization with an Affymetrix Bovine Genome array, which consisted of 24.072 probe sets, each comprising 11 oligonucleotides, covering over 23.000 bovine transcripts. 15.425 probe sets were considered as present by our filtering. The subsequent statistical analysis revealed significant expression differences ($p<0.05$) between infected and uninfected animals for 6.300 probe sets. The resulting data were submitted to the GEO database (GSE13852). Apparently 4.538 transcripts were significantly more abundant in diseased animals as compared to healthy animals. Furthermore, 1.770 transcripts were significantly less abundant in animals with MCF. 1.238 transcripts were found to be more than twice as abundant in diseased than in healthy animals. The highest value of higher abundance (256-fold) was found with a transcript for granzyme-2, a T-cell serine protease, which is transcriptionally activated during cytotoxic T-lymphocyte maturation. The detailed data were deposited in the GEO database (GSE13852).

Inflammation and T-cell activation. As expected from the etiology and the clinical disease picture, primarily transcripts related to inflammatory processes, lymphocyte activation, catalytic processes, immune response, cell proliferation and apoptosis were detected at different abundance. Unexpectedly, the IL-2 transcript was eminent among the transcripts of low abundance. Since IL-2 is strictly regulated on the transcriptional level [31] and because it occupies a pivotal role in the regulation of the immune response and due to the fact that IL-2 deficient mice show a similar phenotype to cattle with MCF, i.e. accumulation of lymphocytes in the intestine and ulcerative colitis [32,33], we suggest that its low abundance in the context of inflammation and T-cell activation may represent a key feature of MCF.

The transcripts for the IL-17 receptor, MHC-I heavy chain, IL-6 receptor alpha chain, insulin receptor, and IL-16 receptor were among the most strongly reduced transcripts. On the other hand, the expression of IL-10 and its receptor were slightly increased. An interesting phenomenon was observed with various clusters of the T-cell receptor (TCR). While the TCR beta cluster (Bt63956) was in low abundance, other beta clusters as well as the gamma cluster were significantly increased (Table 3). A number of transcripts belonging to the effector molecules of cytotoxic T-cells, e.g. granzymes and perforin, as well as transcripts indicating the lymphocytes to be activated, e.g. CD2, CD3, CD8 were detected at higher abundance. The Interferon gamma transcript itself was 6.4 times more abundant in animals with MCF, and in accordance with this we observed that among 21 interferon-related transcripts, 20 were found to be more abundant and only 1 was less abundant. The outstanding higher abundance of transcripts belonging to catalytic processes may be explained by the predominance of cytotoxic T-cells in MCF lesions.

Cell cycle/Apoptosis associated transcripts. Several cell cycle and apoptosis related transcripts were present at different abundance in healthy and MCF-diseased cattle, respectively (Table 4). Interestingly, the transforming growth factor beta (TGF beta) as well as its receptor (TGFbR), which together control proliferation and differentiation of many cell types, was less abundant in animals with MCF. This is noteworthy since TGF beta is, similar to IL-2, important in the context of regulatory T-cells. The complexity of the present situation may be explained by the simultaneous influence of the virus and host control mechanisms on affected lymphocytes.

Overall, these results were consistent with hypothesis 2, which claimed that the host gene expression of animals with MCF was affected in a manner that could be detected by microarray analysis and that could explain the disease phenotype without all dysregulated cells being infected. However, it remains to be clarified, whether these observations are caused by differential gene expression regulation of the cells or by their mere numbers and abundances.

CD4/CD8 ratios

The relative abundance of transcripts in a given compartment may be explained either by downregulation/upregulation of transcriptional activity or by loss/proliferation of the major producer cells of a particular transcript. To address this issue as far as possible, CD4/CD8 ratios were determined in the peripheral blood as well as in the lymphnodes of cattle with MCF and healthy controls. As shown in Fig. 3., the CD4/CD8 ratios in the bloodstream did not significantly differ between healthy animals and such with MCF ($p = 0.97$). In contrast, the same ratios were significantly lower in cattle with MCF compared to healthy controls ($p = 0036$), when assayed in lymphnodes. Due to the nature of the lymphnode, absolute counts of the relevant cells could not be generated. Therefore, the observed low abundance of IL-2 transcripts in lymphnodes of cattle with MCF might be interpreted either as due to (1) over-proportional proliferation of CD8+ cells and downregulation of IL-2 transcription in CD4 cells or (2) strong decline of the CD4+ cells, which are the main IL-2 producers, or (3) a combination thereof. However, it was obvious that the number and fate of lymphocytes in the lymphnodes may differ from the situation in the periphery.

In any event, our results strongly suggest that the low abundance of IL-2 transcripts in lymphnodes of cattle with

Table 3. Transcripts associated with inflammation and T-cell activation affected in lymphnodes of MCF affected cattle.

Transcript	Fold abundance	p-value	Function
Granzyme A	8.6	0.000372	Cytotoxic T-cell effector
Granzyme B precursor	256.2	0.0175	Cytotoxic T-cell effector
Granzyme H precursor	27.2	0.000431	Cytotoxic T-cell effector
Perforin	4.8	0.00517	Cytotoxic T-cell effector
CD2	2.4	0.00569	Adhesion molecule involved in T-cell activation
CD3ε	1.7	0.00517	Essential role in TCR signal transduction and cell-surface expression of the TCR
CD3δ	1.7	0.00837	
CD3γ	1.5	0.00838	
CD8α	3.9	0.00598	Coreceptor for MHC class I restricted T-cell s
CD28	2.2	0.00161	Costimulation of T-cell proliferation and cytokine production upon binding CD80 or CD86
TCR, gamma cluster	4.8	0.0457	Recognition of antigen presentation
TCR, beta cluster (Bt63956)	0.4	0.0364	Recognition of antigen presentation
TCR, beta cluster (Bt1978)	1.4	0.0126	Recognition of antigen presentation
TCR, beta cluster (Bt1978)	1.4	0.00852	Recognition of antigen presentation
Interferon gamma	6.4	0.00958	Affects activation, growth, and differentiation of T-cells, B-cells and macrophages as well as Nk cells. Upregulates MHC expression on APCs. Antiviral and anti-proliferative activities
Interleukin 2	0.14	0.00268	Involved in propagation and establishment of self tolerance of T-cells
Interleukin 7	0.6	0.0277	Growth factor for T-cell progenitors
Interleukin 10	2.9	0.0132	Anti-inflammatory cytokine
IL-10 receptor	1.3	0.007	Anti-inflammatory response

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MCF may lead to IL-2 deficiency and be an important factor in the pathogenesis of the disease.

Discussion

Malignant catarrhal fever (MCF) in cattle is a frequently lethal disease, which proceeds with fever, depression, conjunctivitis and keratitis, as well as with hyperemic to ulcerative lesions in the mucosa of the respiratory, genital and digestive tract, which lead to ocular and nasal discharge and diarrhea [11]. Viral DNA can be detected in buffy coat cells of cattle with MCF as well as in most organs. In contrast, demonstration of viral antigen in cattle with MCF has, thus far, been largely unsuccessful [2,3].

At least two agents, AlHV-1 and OvHV-2, are known to cause MCF in cattle and it would be of interest to know, whether or not their disease is actually based on the same pathogenetical principles. In this work, we concentrated exclusively on OvHV-2. Similar to other gamma herpesviruses, OvHV-2 has two sets of genes, i.e. (1) a set of common viral genes that are conserved among the herpesviruses and (2) a set of unique genes that often have homologues in their host cells. Twelve (Table 1) of its 73 predicted ORFs encode for unique genes, nine (Ov2, Ov3, Ov4.5, Ov5, Ov6, Ov7, Ov8, Ov9, Ov10) have orthologues in AlHV-1 and three are exclusive for OvHV-2 (Ov2.5, Ov3.5, Ov8.5).

It is well known from other gamma herpesviruses that the viral gene expression pattern may correlate to the present state of the infection or the associated disease. For example, the latency II program of EBV drives the differentiation of activated B-cells into memory cells [34,35] or vGCR of HHV-8 is known to induce transformation and angiogenesis, both features, which are required for the development of Kaposi's sarcoma [36,37,38,39].

Although the pattern of viral gene expression in infected cells of animals with MCF has previously not been analyzed in detail, a recent report described the detection of the ORF25 transcript, which encodes for the OvHV-2 major capsid gene, in animals with MCF [40]. This observation spoke for lytic replication of OvHV-2 in diseased animals, at least upon infection with an American OvHV-2 strain. In contrast, we were unable to detect the ORF25 transcript throughout our work, which, though, was done in the context of European strains of OvHV-2. Interestingly, the ORF25 transcript was also not detected, when analyzed by others in the rabbit model for AlHV-1-associated MCF [41]. The latter authors explicitly did not detect any ORF25 (capsid protein) or ORF9 (DNA polymermase) transcripts in spleens or lymphnodes of rabbits with AlHV-1-induced MCF. They concluded, therefore, that AlHV-1-induced MCF is associated with a predominantly latent infection. This view is also shared by others working with European OvHV-2 [42]. Indeed, mainly intact circular OvHV-2 genomes have been found in T-lymphocytes derived from cattle with MCF, which speaks for a dominating latent infection. Furthermore, detection of structural viral antigens in the lesions of cattle with MCF was, hitherto, unsuccessful. Moreover, OvHV-2 is rarely, if at all, naturally transmitted from one cattle to another and it has been difficult to recover infectivity at all from cattle with MCF [43,44,45]. Conversely, a mixture of OvHV-2 transcripts has been detected in cultured peripheral T-lymphocytes from cattle with MCF [29]. Those authors concluded that, at least in the periphery, latently infected cells may co-exist with cells harboring productively replicating OvHV-2. This view is supported by a recent publication on OvHV-2-induced MCF in the rabbit model, where structural OvHV-2 proteins were detected exclusively in epithelial cells and M-cells of the appendix, while other tissues

Table 4. Cell cycle and apoptosis related transcripts affected in MCF in cattle.

Transcript	Protein family	Fold induction	p-value
Cyclin A2	Cyclins	4.2	0.000587
Cyclin B1		7.3	0.000159
Cyclin B2		5.3	0.00247
Cyclin E2		3.7	0.0015
Fas ligand	Membrane bound cytokine	5.1	0.0031
Fas	Tumor necrosis factor	1.4	0.0045
Cyclin dependent kinase 2		2.2	0.00114
Cyclin dependent kinase 5		1.7	0.00458
Cyclin dependent kinase 7		1.5	0.0158
Cyclin dependine kinase 11		1.4	0.0375
BH3 domain interacting death agonist (BID)	B-cell lymphoma type 2 (Bcl2)	2.2	0.000862
Bcl2-associated X protein (BAX)		2.9	0.00503
Bcl2-like 7 (BAK)		2.3	0.00275
Bcl2		1.6	0.00159
Voltage-dependent anion channel (VDAC) (3 probe sets)		2.2	Probe set 1 = 0.00077
		2.0	Probe set 2 = 0.00142
		1.6	Probe set 3 = 0.00186
Baculoviral IAP repeat containing 5 (survivin)	Inhibitors of apoptosis (IAP)	6.3	0.00326
Siva		2.1	0.000791
T-cell specific tyrosine-protein kinase (LCK)	Tyrosine-protein kinase	1.9	Probe set 1 = 0.000703
		2.0	Probe set 2 = 4.23×10^{-5}
		1.8	Probe set 3 = 3.28×10^{-5}
Apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain)		2.4	0.00248
Hsp10	Heat shock proteins (Hsp)	2	0.00158
Hsp70		0.34	0.00833
Hsp90		1.8	8.2×10^{-5}
Retinoblastoma protein (Rb) (2 probe sets)		2.2	9.96×10^{-5}
		1.5	0.004
E2F	Transcription factor	2.2	5.7×10
p53	Transcription factor	1.3	0.00134
TGF beta	TGF beta family	0.29	0.006
TGF beta receptor		0.35	0.01

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contained viral DNA at detectable levels but not the corresponding mRNAs or proteins [26].

However, in lymphnodes of cattle with MCF, as demonstrated throughout our experiments, neither genes encoding for structural proteins nor any one of the unique genes of OvHV-2 were detected above threshold levels. Indeed, the only active gene seemed to be ORF73. Detection of this RNA was confirmed in our work by three different approaches. Interestingly, ORF73 (LANA) transcripts were also detected in rabbits with AlHV-1-induced MCF [41]. Thus, our data with OvHV-2 in cattle match very well with the observations described for AlHV-1 in rabbits. Together, these data imply that MCF, independent of its individual agent or host, is associated with a predominantly latent viral gene expression pattern, at least in certain tissues.

In addition, we detected by microarray a transcriptional activity in a region without any predicted ORF. The existence of this transcript was confirmed by qRT-PCR over the same region (Table 2). Although the sensitivity of the qRT-PCR for this transcript was below that for ORF73, the number of molecules covering the intergenic region was higher than the number of ORF73 transcripts. Since the detection limits of the assays for ORF25 and intergenic RNA were comparable, the detection of the latter RNA also corroborates the absence of the former.

While a latency pattern, rather than a replicative pattern, of viral gene expression could be expected in the context of MCF, the lack of expression from the atypical genes argued against our first hypothesis, which claimed that some of those genes and their products might directly drive survival of the dysregulated cells. One might predict that those genes and their products have a function in protecting their original host from developing MCF. Absence of their expression in cattle could even be one of the major reasons for the development of MCF. Unfortunately, OvHV-2 gene expression could not yet be determined in ovine cells, primarily due to the scarcity of infected cells in sheep (own unpublished observations).

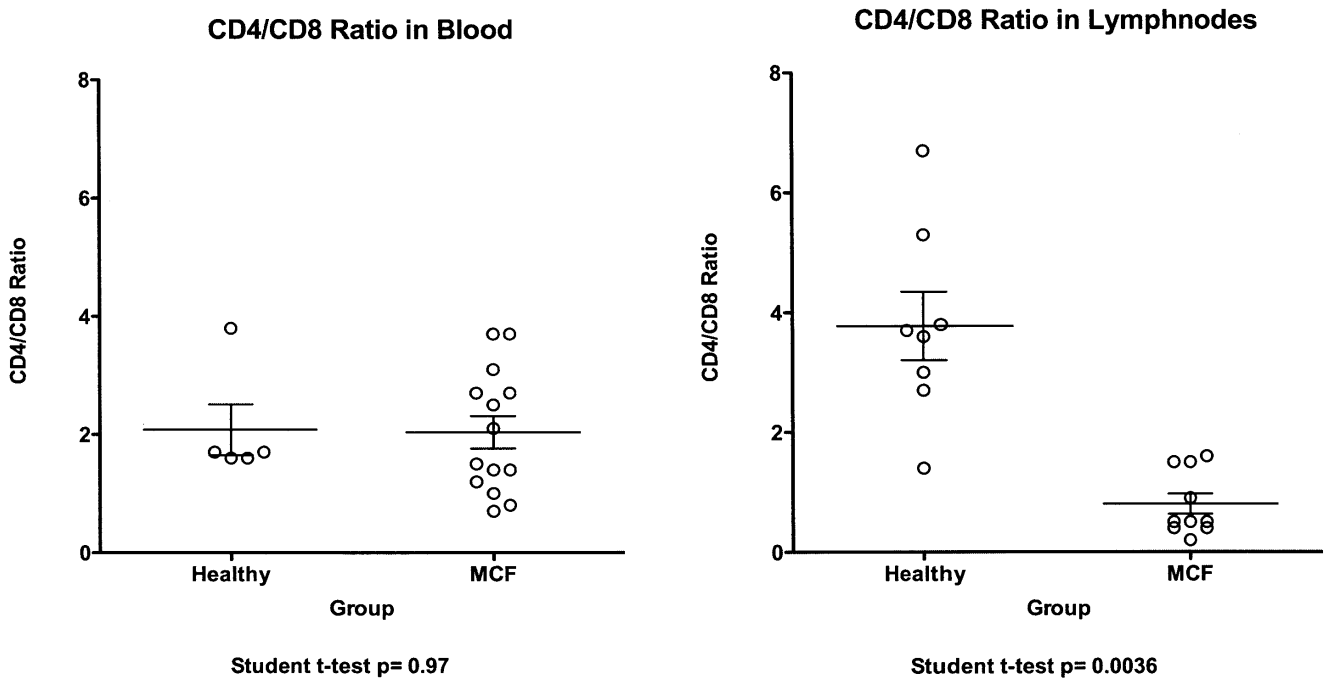

Figure 3. CD4 to CD8 ratios. The CD4 to CD8 ratios (y-axis) in blood (left panel) and inguinal lymphnodes (right panel) of healthy animals and cattle with MCF are shown. Open circles indicate individual values, horizontal bars give median values, and standard deviations are shown by vertical brackets.
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Host gene expression

The host gene expression profile was markedly disturbed by the effects of the disease. One may argue, whether or not the lymphnodes are the ideal tissue to look for viral and host gene expression throughout MCF. They were selected for the present study because they are known to harbor plenty of OvHV-2 infected cells, which play an important role in the host's immune defense [10,22]. Moreover, the vasculitis and necrosis in affected tissue have been attributed to the function of riotous cytotoxic lymphocytes. It was not surprising to find a complex pattern of differences in transcript abundances upon comparison of transcriptomes in lymphnodes from healthy cattle and cattle with MCF.

High abundance of Cyclin and Cdk transcripts

The state of cell proliferation can be monitored through analysis of gene expression and was reflected on the present microarray. As the cells progress through the reproductive cycle, cyclins are synthesized and degraded. Cyclins bind to and, thus, activate cyclin-dependent kinases (Cdks). Activated Cdks induce expression of more cyclins as well as degradation of the Cyclin-Cdk inhibitor of the consequent step in the cell cycle. In contrast to cyclins, Cdk levels remain, under normal conditions, constant throughout the cell cycle. However, cyclin-dependent kinases are frequently upregulated in malignancies due to overexpression of their cyclin partners [46]. Thus, as a first marker of malignancy, a significant higher abundance of several cyclins as well as Cdk was detected in the lymphnodes of cattle with MCF (Table 4). This finding could be expected since histology of lymphnodes in MCF diseased cattle is dominated by a marked increase in lymphocytes [47], although foci of necrosis are concurrently detected [16,17].

Activation of T-cells and unbalanced expression of regulators of apoptosis

T lymphocyte activation can be expected upon a viral infection of an organism or during defense against uncontrolled cell growth. Indeed, several markers of T lymphocyte activation were more abundant in cattle with MCF as compared to healthy animals (Table 3). To avoid accumulation of excessive amounts of activated T-cells, both the extrinsic and the intrinsic apoptosis pathways will usually be induced, simultaneously with T-cell activation. Both pathways lead to the activation of a family of cystein proteases called caspases. Caspases are constitutively present in most cells as inactive proenzymes and are activated by specific proteolytic cleavage. On a transcriptomic microarray, changes in apoptosis levels will therefore likely be reflected by changes in the levels of transcripts of apoptosis regulatory proteins without directly affecting the transcript levels of the caspases themselves. In the present study, the expression of antiapoptotic members of the Bcl-2 family as well as other inhibitors of apoptosis (IAP), were more abundant in cattle with MCF than in healthy animals (Table 4).

However, opposing forces, stimulating cell death, were also observed in lymphnodes of cattle with MCF. Death receptors are cell surface receptors that send apoptosis signals to the inside of the cell when they are bound by death ligands. Death receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily. They all contain a homologous cytoplasmic sequence named death domain. The best characterized death receptors and corresponding death ligands are Fas (Fas and its ligand FasL, also called CD95 and CD95L) and TNF (TNFR1 and its ligand TNF) [48]. T-cell receptor (TCR) engagement, i.e. activation of T lymphocytes leads to the expression of Fas (CD95) on the surface of the activated T-cell, a prerequisite to undergo activation induced cell death (AICD) [49,50]. Binding of FasL to Fas results in trimerization of Fas, which includes the approximation of death domains in the cytoplasmic tails of Fas. In the present study, the FasL as well as Fas transcripts were detected at significantly higher abundance in cattle with MCF (Table 4). FasL activity was previously described to be induced predominantly at the transcriptional level [51,52,53]. CD27 is another member of the

TNF receptor family expressed on B and T-cells. Siva, which was found to be highly abundant in the context of MCF (Table 4), is an intracellular ligand of CD27. It is expressed in lymphoid cells and exhibits proapoptotic activity [54,55].

In addition, some tumor suppressor genes, i.e. retinoblastoma protein (Rb) and LCK as well as the proapoptotic p53 were more abundant in lymphnodes of cattle with MCF than in healthy cattle (Table 4). Rb is known to arrest cells in the G1 phase of the cell cycle by binding to E2F transcription factors (Reviewed by [56], while free E2F factors are important for the induction of S phase entry. Complexes of Rb family members and E2Fs recruit histone deacetylase and other chromatin remodeling factors to E2F-responsive promoters and therefore inhibit transcription from the same [57,58,59]. However, E2F transcripts were also more abundant, which might counter balance the arresting effect of Rb.

In T lymphocytes protein tyrosine kinases (LCK) play an integral role in the activation of cells through various immunoreceptor molecules [60,61]. Apart from the activation of the cell cycle, LCK have been shown to be involved in programmed cell death of T lymphocytes. Gonzalez-Garcia et al showed an induction of CD95 ligand through LCK [62]. Samraj et al showed a positive regulation of the mitochondrial apoptosis by LCK [63]. In our experiment LCK was highly more abundant in the context of MCF.

Thus, both pro- and anti-apoptotic forces seemed to be induced in cattle with MCF, which might lead to a pathogenic alteration in the natural balance between cell survival and cell death.

Low abundance of the IL-2 transcript may explain the disease phenotype

IL-2 is used as an essential factor for the propagation of T-cells in culture [64]. Based on this property, IL-2 was also used for the augmentation of T-cell responses in vivo in cancer or AIDS patients [65,66]. Importantly, IL-2 is strictly regulated at the mRNA level, which depends on signaling from the TCR and CD28. The suitability of both, microarray and real-time RT-PCR, for determining the levels of IL-2 mRNA in bovine cells and the good correlation of the two methods has been confirmed in a different context by others [67]. In our analysis, a significant low abundance of the IL-2 transcript was noticed in cattle with MCF, while CD28 and TCR transcripts were present at higher than normal abundance. Since stimulation through CD28 in addition to the TCR can provide a potent co-stimulatory signal to T cells for the production of various interleukins (IL-2 and IL-6 in particular), these observations imply that MCF is associated with a low abundance of the IL-2 transcript.

However, the low abundance of the IL-2 transcript might be due either to down regulation of its expression or to the loss of IL-2 producer cells. It was, therefore, interesting to note that the ratio of CD4+ to CD8+ T-cells in lymphnodes of cattle with MCF was decreased when compared to healthy controls. Interestingly, this same change has been observed in the AlHV-1-based rabbit model [41]. Yet, there, the change was associated to an increased growth of CD8+ T-cells, which in turn cannot explain any low abundance of the IL-2 transcript. Therefore, we like to speculate that down regulation of IL-2 transcription may at least be partially responsible for low abundance of the IL-2 transcript in cattle with MCF.

Of note, the ratios between CD4+ and CD8+ T-cells were unaffected in the periphery, when analyzed in the bloodstream of cattle. This was in contrast to the observations made in rabbits with AlHV-1-induced MCF [41]. This is inasmuch important that the ratio measured in the periphery does not necessarily reflect the picture that is found in the lymphoid organs.

Interestingly, mice lacking a functional IL-2 system develop largely normal until the age of 4 to 6 weeks, where they start to suffer from polyclonal expansion of T- and B-cells. This expansion causes enlargement and non-purulent inflammation of lymph nodes, spleen, and gut-associated lymphoid tissue due to accumulation of activated T-cells. Similarly, a human patient with IL-2 receptor deficiency showed also signs of T-cell abnormalities as evidenced by lymphadenopathy, chronic inflammatory disorders, and lymphocytic infiltration of multiple organs [68]. Furthermore, T-cells from mice lacking either IL-2 or the IL-2 receptor have been reported to be resistant to activation-induced cell death in vitro and in vivo. Alternatively, it has been proposed that the abnormal growth rate of T-cells in association with IL-2 deficiency may be due to tolerogenic properties of IL-2, mediated through interactions with regulatory T-cells (Treg)[69,70,71]. Since IL-2 in the lymphnode is critical for the development and peripheral expansion of Treg (CD4+CD25+), which promote self-tolerance by suppressing autoreactivity of T-cells as well as limiting T-cell replication in vivo (reviewed by [31], decreased IL-2 levels may explain the accumulation and autoreactivity of the T-cells in MCF. In any event, the disease signs and abnormal T-cell properties seen in mice and men without functional IL-2 system are very reminiscent of the phenotypes associated to MCF in cattle. Thus, it seems that lack of IL-2 may play an important, if not central role in the development of MCF.

These observations instigate hope that it might be possible to treat MCF in cattle by supplementing IL-2. One might argue that it would be desirable to measure the peripheral IL-2 concentrations in order to support or reject this hypothesis. However, it has to be kept in mind that IL-2 functions mainly in the lymphnodes and peripheral IL-2 concentrations do not necessarily correctly reflect the micro situation in the lymphnode. Indeed, it has been shown by others that IL-2 supplementation may have considerable effects on the immune responses, while measurement of peripheral IL-2 expression and applied amount of external IL-2 were not a good indicators for its function *in vivo* [72,73].

Potential roles of OvHV-2 proteins and transcripts in the development of MCF

Latency-associated nuclear antigens (LANA of HHV-8 or SaHV-2 and EBNA-1 of EBV) have been shown to play important roles in the development of gamma herpesvirus-associated neoplastic diseases in humans and other primates. However, their mode of action is associated with the expression levels or the functions of some major protooncogenes. For example, LANA of HHV-8 may either repress transcription of p53 [74] or direct the p53 protein to proteasomal degradation [75]. Furthermore, it can bind and inactivate retinoblastoma protein (Rb), thereby transactivating E2F transcription [76]. Similarly, SaHV-2 LANA can interfere with p53 or Rb functions, while EBV EBNA-1 interferes with p53 and HAUSP (Herpesvirus associated ubiquitin-specific protease) [77].

OvHV-2 ORF73 has been predicted to encode for a LANA orthologue, although its function has not yet been demonstrated [27]. LANAs are supposed to bind to the origin of latent viral DNA replication (oriP) and tether the viral DNA to the host's chromosome in order to allow co-replication of the viral genome with the cellular genome upon mitosis. For this purpose, any typical LANA orthologue needs to have DNA-binding properties, which might explain interference with the host's gene expression profile. Thus, binding of the LANA protein to any locus within the host's chromosome, including the IL-2 locus, might affect the patterns of gene expression, including IL-2 expression. Alternatively, the LANA protein might undergo interactions with host proteins, similar to the interactions

described for its orthologues in other viruses. It will be interesting to analyze these possibilities in the future.

The second transcriptionally active region detected in cattle with MCF spanned nucleotides 115184 to 115364 bp on the forward strand of the viral genome. At present, one may only speculate about the nature and significance of this transcript. According to our results from qRT-PCR, this signal is attributable to one transcript that may even further extend into both directions. Although no STOP codon is evident in the +3 reading frame within the minimal boundaries of this transcript, it does not necessarily represent a hitherto undetected gene. According to its sequence, it may form a hairpin structure, which can be found in micro RNAs. Thus, it may be similar to BARTs and EBERs of EBV or to micro RNAs, which have been described for other viruses, including many herpesviruses [78,79,80,81]. Such RNAs might interfere with the host's gene expression through mechanisms like siRNA or other means of silencing.

It will be interesting to address these issues in consecutive studies. Furthermore, it remains to be established, whether or not these observations are also true for MCF in animals other than cattle.

Conclusions

We have determined the major viral gene expression pattern in lymphnodes of cattle with MCF. Only two sites were transcriptionally active, one with the potential to express ORF73, a LANA-orthologue, the other with some likelihood to represent a thus far unrecognized OvHV-2 gene or, maybe, miRNAs. Overall, this viral gene expression profile is similar to the one found in the context of AlHV-1-associated MCF in the rabbit model [41]. The prominent counterpart to this latent gene expression pattern on the host's side was a significantly lower abundance of IL-2 transcripts, an increase in lymphocyte activation, as well as increase and decrease in apoptosis associated transcripts in lymphnodes of cattle with MCF as compared to the same tissue from healthy animals. Taken as a whole, these results were consistent with one of our hypotheses, which claimed that the host gene expression of animals with MCF was affected in a manner that could be detected by microarray analysis and that could explain the disease without all dysregulated cells being infected. Since the phenotype of mice with deficient IL-2-system almost perfectly matches the colitis observed in cattle with MCF, we suggest that OvHV-2-linked low abundance of IL-2 transcripts may be a key to further study the pathogenesis of MCF. Clearly, in the present study, we did not discriminate between intracellular regulation of IL-2 mRNA expression and relative depletion of the lymphnode to harbor IL-2 producing cells. This important differentiation will have to be addressed in future studies. However, MCF may be looked at as an infectious form of IL-2-deficiency, which occurs as a natural disease of animals.

Materials and Methods

Animals

Mediastinal lymphnodes were taken from cattle at the slaughter house (11 healthy controls) and from 6 naturally diseased animals originating from conventional Swiss cattle farms, which had to be euthanized due to MCF. In addition, EDTA blood samples as well as inguinal lymphnodes were collected from 10 cows with and 8 cows without MCF.

Construction of the OvHV-2 Agilent custom 11 k microarray

Based on the published sequence of OvHV-2 [27] we designed an oligonucleotide microarray for the analysis of viral gene expression in MCF. 60mer probes were chosen to match stretches along the entire genome [27] starting at genomic position 823 with an interval of 30 nucleotides, such that every position was covered by two probes. This was done for the forward as well as for the reverse strand. Further, we selected two more probes for each predicted open reading frame (ORF) of OvHV-2. These probes were designed using the software Arraydesigner (Premier Biosoft, Palo Alto, USA) and chosen such that the probe length was between 55 and 60 nucleotides and the predicted melting temperature was between 75°C and 80°C. The chip description was deposited at GEO database (GPL7746). Only probes with a quality score of 'good' were used. Altogether, we designed 8.876 probes targeting the viral genome. Additionally, we included 1500 oligonucleotides corresponding to known cattle genes for primary normalization of the hybridization intensities. Using our probe sequences, we ordered 11 k custom microarrays from Agilent. Microarrays were produced by Agilent by *in situ* synthesis technology.

Affymetrix microarray for bovine transcription profile

We used the GeneCHIP® Bovine Genome array (Affymetrix., P/N 900561) for the analysis of the host gene expression profile.

Total RNA preparation

Total RNA was isolated from lymph nodes of 9 cattle, two of which were sacrificed due to MCF, the others were MCF negative. Virus status of all animals was confirmed by real-time PCR. Lymph node tissue was frozen in liquid nitrogen and homogenized using a mortar and pistil. Total RNA was isolated using the RNeasy Kit by Qiagen (order number 74106, RNeasy, Qiagen, Hombrechtikon, Switzerland). Immediately upon homogenization the samples were taken up in RLT buffer with 1% β-mercaptoethanol (Sigma, Buchs, Switzerland). DNA was digested using RNase-free DNase (Qiagen, order number 79254) at room temperature for 15 minutes. RNA concentration was measured using a Nanodrop 1000 (NanoDrop Technologies, Delaware, USA). The quality of each sample was checked by a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only those samples with 260 nm/280 nm ratio between 1.89–2.13 and a 28 S/18 S ration within 1.5–2 were further processed.

For the purpose of RT-PCR and qRT-PCR, the extracted RNAs were additionally subjected to removal of contaminating DNA using the Ambion Turbo DNA-free Kit (Applied Biosystems, Rotkreuz, Switzerland).

Fluorescent cRNA preparation for the Agilent microarray (viral gene expression profile)

1.6–5 µg of total RNA were reverse transcribed to cDNA and amplified and labeled to cRNA with the Agilent Low RNA Input Linear Amplification Kit PLUS (order number 5184–3525, Agilent). Briefly, 1.2 µl T7 Promoter Primer and 1 to 5 µg of total RNA in a total volume of 11.5 µl were denatured at 65°C for 10 minutes. Then the reaction was placed on ice for five minutes. After that 8.5 µl cDNA master mix consisting of 4 µl 5× first strand buffer, 2 µl 0.1 M DTT, 1 µl 10 mM dNTP mix, 1 µl MMLV reverse transcriptase and 0.5 ul RNaseOUT were added to each sample and incubated at 40°C for 2 hours. Subsequently the enzyme was heat inactivated at 65°C for 15 minutes. Then the samples were placed on ice for five minutes. For the synthesis of fluorescent cRNA 2.4 µl Cyanine 3 or Cyanine 5-CTP (10 mM) and 57.6 µl transcription master mix consisting of 15.3 µl nuclease free water, 20 µl 4× transcription buffer, 6 µl 0.1 M DTT, 8 µl NTP Mix, 6.4 µl 50% PEG, 0.5 µl RNaseOUT, 0.6 µl inorganic

pyrophosphatase and 0.8 µl of T7 RNA Polymerase were added and the reaction was incubated at 40°C in the dark for 2 hours. Labeled cRNA samples were isolated again with the RNeasy Kit (Qiagen).

Hybridization of Agilent microarrays

From each positive animal, a Cy3- and a Cy5-labeled RNA sample, respectively, was co-hybridized with a reference sample with the opposite labeling and used as dye-swap pairs. As reference, the pool of the seven OvHV-2 negative samples was used. The specific activity of all samples as concentration of dye (pmol/µl) divided by concentration of RNA (µg/µl) was calculated. For fragmentation and hybridization we used the Agilent Gene Expression kit for oligo microarrays (Agilent, order number 5188–5242, protocol G4140-90050). Before hybridization the chips were blocked using the 10× blocking agent provided with the kit. 350 ng of Cy3 labeled RNA and 350 ng of Cy5 labeled RNA were mixed with 20 µl 10× blocking solution (provided with the kit), 4 µl 25× fragmentation buffer and toped up with nuclease-free water to 100 µl. Fragmentation was performed at 60°C in a hybridization oven (Agilent, G2545A) in the dark for 30 minutes. 100 µl 2× hybridization buffer was added to each tube and the microarray and gasket slide were assembled. The slides were hybridized at 65°C, for 17 hours at 4 rpm in a hybridization oven. After the hybridization, slides were washed twice for 1 minute in wash solution 1 (6× SSPE (Sigma, order number S2015), 0,5% N-lauroylsarcosine (Sigma, order number L7414) in de-ionised, nuclease-free water). Then the slides were transferred to wash solution 2 (0.06× SSPE, 0.5% N-Lauroylsarcosine) and incubated 1 minute. After this, the slides were transferred to an acetonitrile bath (Sigma, order number A3396) and incubated for 1 minute. At last the rack was transferred to Agilent stabilization and drying solution (Agilent, order number 5185–5979) and incubated for 30 seconds.

Scan and Data analysis of Agilent microarrays

Microarray slides were scanned with an Agilent microarray scanner and the scans were quantified with the Agilent Feature Extraction software 8.5.1.1. Background subtraction and dye normalization for each array was performed within the Agilent Feature Extraction software with default settings. The quantified data was subsequently loaded into GeneSpring 7.3.1 for further analysis. Expression data from the dye-swap pairs were averaged in order to eliminate any potential gene-specific dye effect. Probes with signals close to background (average signal<500) as well as probes which were flagged as saturated by the Agilent Feature Extraction Software were excluded. From the remaining probes the average ratio of MCF positive vs MCV negative animals was computed.

Real-time PCR

The Taqman real-time PCR for detection of OvHV-2 DNA in animal samples was used essentially as described previously [23]. Primers and probe are listed in Table 5.

qRT-PCR

Quantitative two-step RT-PCRs were established (specificity, sensitivity, efficiency) for ORF73, Ov9, and intergenic RNA as described previously [82]. Briefly, RNA was extracted and purified from contaminating DNA as described above. The QScript cDNA Supermix (Quanta Biosciences, VWR International, Dietikon, Switzerland) was used for cDNA synthesis (30 min at 42°C before inactivating the RT at 85°C for 5 min) in a volume of 20 µl. For control reactions the same amounts of RNA template were diluted to the same volume in RNase/DNase-free water and kept on ice during the RT cycle. In the second step, 1 µl of cDNA (or RNA) was mixed with 10 µl Perfecta SYBR Fastmix (Quanta) and appropriate amounts of primers (100 nM final concentration; Table 5) before being brought to a volume of 20 µl. The following cycles were run to yield a detection limit of between 10 and 100 copies per sample: 10 min at 95°, 40 cycles of 5 sec at 95° and 20 sec at 60° (only 58.3° for ORF73). The melt curve was run immediately after amplification, starting at 50°C and increasing the temperature for 80 cycles by 0.5°C every 10 seconds. Sensitivity and efficiency were established by using decreasing concentrations of cloned DNA templates, whereas controls for specificity included templates from unrelated viruses [82,83].

Table 5. Oligonucleotides used.

Oligonucleotide	Sequence (5′ to 3′)
OvHV-2 forward	TGG TAG GAG CAG GCT ACC GT
OvHV-2 reverse	ATC ATG CTG ACC CCT TGC AG
OvHV-2 probe	[6FAM]-TCC ACG CCG TCC GCA CTG TAA GA
LANA2-F	GTG GAG CGT TAG GAT TGA GC
LANA2-R	CAG GGC AAA ACG TAA AAA GC
Ov9-F	CGG GAC CAT TAC AAG AAG
Ov9-R	GCA TAA CAG AAG CAT AGC
Intergenic-F	GTG TGG TGA CAC ATT CCC AG
Intergenic-R	ATG TAA GAC CCC TTA GCC CC
ORF25-F	ACT GCG GAC GTG GCC TAC TT
ORF25-R	GTC CAG GAG GGC TCG GTG TG

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Conventional RT-PCR

Conventional RT-PCR for ORF25 was performed as described by others [40]. The templates and controls used here were the same as for qRT-PCR described above.

cRNA preparation for the Affymetrix microarray

Total RNA samples (2 µg) were reverse-transcribed into double-stranded cDNA, in vitro transcribed in presence of biotin-labeled nucleotides using a IVT Labeling Kit (Affymetrix Inc., P/N 900449, Santa Clara, CA), purified and quantified using BioRobot Gene Exp–cRNA Target Prep (Qiagen AG, Switzerland). The labeled cRNA quality was determined using a Bioanalyzer 2100.

Hybridization of the Affymetrix microarray

Biotin-labeled cRNA samples (10 µg) were fragmented randomly to 35–200 bp at 94°C in Fragmentation Buffer (Affymetrix inc., P/N 900371) and were mixed in 300 µl of Hybridization Mix (Affymetrix Inc., P/N 900720) containing Hybridization Controls and Control Oligonucleotide B2 (Affymetrix Inc., P/N 900454), before hybridization to GeneCHIP® Bovine Genome arrays (Affymetrix Inc., P/N 900561) for 16 hours at 45°C was performed. Arrays were then washed using an Affymetrix Fluidics Station 450 (FS450_002 protocol. An Affymetrix GeneChip Scanner 3000 (Affymetrix Inc.) was used to measure the fluorescent intensity emitted by the labeled target.

Statistical analysis of the Affymetrix microarray

Raw data processing was performed using the Affymetrix GCOS 1.4 software (Affymetrix Inc.). After hybridization and scanning, probe cell intensities were calculated and summarized

for the respective probe sets by means of the MAS5 algorithm [84]. To compare the expression values of the genes from chip to chip, global scaling was performed, which resulted in the normalization of the trimmed mean of each chip to a target intensity (TGT value) of 500 as detailed in the statistical algorithms description document of Affymetrix (Affymetrix, 2002, see also [85]). Quality control measures were considered before performing the statistical analysis. These included adequate scaling factors (between 1 and 3 for all samples) and appropriate numbers of present calls calculated by application of signed-rank call algorithm [86]. The efficiency of the labeling reaction and the hybridization performance was controlled with the following parameters: present calls and optimal 3′/5′ hybridization ratios (around 1) for the housekeeping genes (GAPDH and ACO7), for the poly A spike in controls and the prokaryotic control (BIOB, BIOC, CREX, BIODN).

Differential transcript abundance was identified as follows: Probes that were absent in the uninfected and the infected animals were excluded. We considered a probe absent if it had more than one absent call among the infected animals and more than one absent call among the uninfected animals. Student's t-test was applied to test the present probes for significant infection-induced higher or lower abundance. The magnitude of the change was computed from the averaged values of the infected and uninfected animals.

Isolation of lymphocytes

For the isolation of lymphatic cells from lymphnodes, fat and connective tissue were removed and the remaining tissue was cut into small pieces and filtered through a sieve with a mesh size of 1 mm before being suspended in phosphate buffered saline solution (PBS). After washing tree times with 50 ml PBS, low speed centrifugation, and re-filtering for removal of aggregates, the cells were resuspended in 10 ml PBS and filtered trough a cell strainer with a mesh size of 100 µm (BD Falcon, Bedford, MA, USA).

Fluorescence-activated flow cytometry

CD4 and CD8 subsets were determined in combination with CD2 staining. Anti bovine CD4 (CACT138A), anti bovine CD8 (CACT80C) and anti bovine CD2 (16-1E10) were from VMRD, Inc, Pullmann, WA, USA). For staining, 100 µl EDTA blood or 100 µl isolated lymphnode cells (10^6) were added to 5 µl of pre-diluted antibody (1/100). After incubation for 30 min at 4°C, 2 ml of erythrocyte lysing solution (8.29 g/l NH_4Cl, 1 g/l $KHCO_3$, 37 mg/l Na_2 EDTA) were added. After 3 minutes at ambient temperature, cells were pelleted by centrifugation (350×g). The supernate was discarded and the cells resuspended in 200 µl of PBS supplemented with 1% fetal calf serum (FCS) and the secondary antibodies (APC labeled anti mouse IgG1 from BD Pharmingen, BD Biosciences, San Jose, CA, USA and goat anti mouse IgG2a-FITC from Southern Biotech, Birmingham, AL, USA) diluted 1/1000. After 30 min at 4°C, cells were washed with 2 ml PBS and resuspended in 250 µl PBS with 1% FCS. Finally, cells were analyzed in a FACScalibur (BD Biosciences, San Jose, CA, USA). A gate was set to the region corresponding to the lymphocytes, based on the forward and side scatter diagram. A minimum of 1000 gated events were acquired and analyzed. FL1 and FL4 double positive cells were counted.

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Author Contributions

Conceived and designed the experiments: CSMT HR MF MA. Performed the experiments: CSMT MF AP. Analyzed the data: CSMT HR MF MA. Contributed reagents/materials/analysis tools: AP UW MA. Wrote the paper: CSMT HR MF MA. Revised paper: MA CSMT HR MF AP UW.

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5 Conclusions and perspectives

Substantial new information about OvHV-2 and MCF could be made during this PhD thesis, which broadened our understanding of the disease and also raised many new questions. The salient features of the present work are as follows:

i. OvHV-2 seems to target the same cell populations in sheep (which do not succumb to MCF) and cattle (which succumb). Thus, a differing cellular host range in MCF-susceptible or resistant species seems not to be a crucial factor in the disease pathogenesis. In sheep, the virus targeted first the CD4+ T-lymphocytes and peaked only later the CD8+ cells. Although similar experiments have not been made in cattle, it seems that the CD4+ cells are lost during the first stage of MCF, whereas the CD8+ cells remain and harbor the major load of OvHV-2 DNA. Therefore, the virus may target functionally similar cells in the different animal species but behave differently in them and in a species-specific way.

ii. The predominant state of OvHV-2 throughout MCF seems to be latent, with a most restricted viral gene expression profile, at least in lymphnodes of cattle with MCF. There, only two transcriptionally active loci were detected by microarray analysis, i.e. the ORF73 locus and an intergenic sequence, where no viral gene had been predicted. However, from our experiments in rabbits, we learned that certain tissues, i.e. the appendix of rabbits with MCF, contain structural antigens. This was demonstrated by immunohistology through detecting capsid protein as well as tegument protein of OvHV-2 in epithelial cells and M-cells of the appendix from rabbits with MCF. Thus, MCF seems also to be associated with limited virus replication, specifically in selected tissues.

iii. The loss of IL-2 mRNA and TGFβ mRNA were the most striking features among the thousands of changes that were observed by microarray in the host gene expression profiles of animals with MCF compared to healthy controls. Together with the silence of the viral IL-10 gene (Ov2.5) in animals with MCF, our data suggest that a severe lack, physical or functional, of regulatory T cells may be crucial for MCF pathogenesis. This possibility opens a new and exciting field of research and helps to draw a new picture of MCF and its pathogenesis.

Based on these insights, new aspects can be added to the current picture of OvHV-2 biology:

OvHV-2 in sheep. As previously shown, OvHV-2 circulates among sheep without causing disease [18,58,59,60,61,63]. Most often, the virus enters the organism through the respiratory route, where it replicates before targeting the lymphocytes. CD4+ lymphocytes are targeted first because a peak of viral DNA is found initially in this cell population. Only later, CD8+ lymphocytes accumulate OvHV-2 DNA [31]. At times, OvHV-2 is redirected to respiratory tissues, where it replicates and from where it is excreted, probably in the form of aerosols, in order to be transmitted to a new generation of hosts [33,34,36]. The observation that virus harvested from respiratory tissues is non-infectious upon intravenous application but very much infectious by intra nasal nebulization, suggests that OvHV-2, similar to other gammaherpesviruses, is able to form viruses with different tropisms [4,37]. This issue certainly needs further investigation. The state of OvHV-2 in sheep lymphocytes is, most likely, predominantly latent. It had been in our original planning to address this issue using sorted sheep PBMC. Unfortunately, the quality of extracted RNA did not match the requirements for such an analysis. However, preliminary data by real-time RT-PCR indicate

that at least some of the Ov genes are active in sheep lymphocytes (Meier-Trummer & Ackermann, ongoing work). From the present knowledge, we hypothesize that this may be of crucial importance for maintaining health in the infected sheep. We are convinced that the Ov genes do not merely represent viral luxury but that they have a very important biological function, which just needs to be identified.

OvHV-2 and MCF. Infectious OvHV-2 can be harvested throughout a very narrow time window from nasal epithelia of newly infected sheep [36]. Such infectivity can be used to transmit OvHV-2 by intranasal nebulization to all sorts of susceptible animal species, i.e. sheep, rabbit, cattle, bison. Rabbits, cattle, and bison develop MCF upon such inoculation, whereas sheep remain healthy, unless inoculated with an overwhelmingly high dose of the virus [32]. Very little is known about what happens between the time of OvHV-2-inoculation and the time of development of MCF. According to a recent model in AlHV-2-associated MCF, the virus replicates initially in unidentified tissues before spreading to CD8+ T-cells or their progenitors. There, it establishes a predominantly latent infection, which is suggested to deregulate the infected cells, leading to their proliferation and cytotoxic behavior [92]. Moreover, our own data suggest that OvHV-2 may cause MCF in a very similar manner. Indeed, a potential driving force for this has meanwhile been identified: the RNA transcribed from the non-coding region of OvHV-2, which was first detected throughout the present work, is expressed as a multiple spliced molecule, whose introns have strong characteristics of a miRNA (Uster & Ackermann, ongoing work). It will be of utmost interest to test the hypothesis that such a miRNA has a pivotal role in the pathogenesis of MCF. It is possible that it could influence the biology of T-lymphocytes. Two very attractive possibilities may be: (1) persistent activation of infected CD8+ lymphocytes and (2) resistance of activated CTLs against the action of Treg.

Co-evolution and MCF. It is not known for how long OvHV-2 has circulated among and co-evolved with sheep. However, sheep that may fall to lethal OvHV-2-associated diseases have probably died out a long time ago. Similarly, OvHV-2 that is virulent for sheep died out as well. Nomads have domesticated sheep some 10'000 years ago. In contrast, settlers domesticated cattle but only several thousand years later. Moreover, cattle and sheep were kept preferentially separate until very recently. Consequently, cattle and other animal species that succumb to MCF have had much less time than sheep for participating in the co-evolutionary process with OvHV-2. In this context, it is interesting to note that in his second voyage to the Americas, Columbus carried the Churra breed of sheep on his ships. Thus, it was not before this time that the American bison might have first encountered OvHV-2. Hence, bison had even less time for co-evolution with sheep than cattle. Most interestingly, bisons are also much more susceptible to MCF than cattle [54]. Uptake and maintenance of the Ov genes into the OvHV-2 genome would not make sense if they were not expressed at times and if they did not serve an important biological function. On one hand, it seems that OvHV-2 has taken up and, probably, modified the Ov genes in a manner that prevents disease in sheep, while still allowing for constant circulation of OvHV-2 among sheep. On the other hand, the Ov genes are apparently silent throughout MCF. Therefore, it is attractive to speculate that the Ov genes serve to maintain the health of sheep throughout infection. Thus, they seem to represent attenuation factors rather than virulence factors. However, such a model alone would not explain the occurrence of MCF. Forces that drive the disease must still be present. In order to be tolerated in the sheep's organisms, OvHV-2 has to impede or modulate the host's adaptive immune system. This driving force seems to be counterbalanced by activities provided from the Ov genes and absence of counterbalancing may explain emergence of MCF. At present viral miRNAs as postulated throughout this study represent hot candidates for providing the forces driving MCF.

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